Identification of Host Immune Mechanisms Involved in Enterococcal Infections

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1. Introduction

1.1. *Enterococcus faecalis*

Enterococcus faecalis was first described at the end of the 19th century by Thiercelin as coccoid bacteria present in the intestinal tract [1, 2]. In the same year, Maccallum and Hastings sampled and described an organism that was responsible for a lethal case of endocarditis. This organism is nowadays known as *Enterococcus faecalis*. This finding coincidently provided a first detailed description of its pathogenic capabilities [3]. The genus *Enterococcus* was first described by Schleifer and Klipper-Bälz in 1984 [4, 5]. The name refers to *entero* (gut) and *coccus* (berry). Until 1984 enterococci were classified as *Streptococcus* Lancefield group D. At the beginning only two species were categorized into the new genus of *Enterococcus*, *Enterococcus faecium* and *Enterococcus faecalis*, formerly referred to as *Streptococcus faecalis* or *Streptococcus faecium*, respectively.

For many years species of *Enterococcus* have been underestimated as a causative agent for infective diseases in both humans and animals. Recently, the numbers of infections caused by this pathogen in particular in intensive care units (ICUs) is increasing [6].

Enterococci are Gram-positive, catalase-negative, non-spore-forming and facultative anaerobic bacteria, which normally grow as diplococci or short chains of cocci. Importantly, enterococci are a major problem in hospitals and other medical units [7]. Enterococci are able to survive a wide range of stresses and hostile environments, including those of extreme temperature (5–65°C), a wide range of pH-values (4.5-10.0) and high sodium chloride concentration. This unique tolerance to extreme conditions enables them to colonize a wide range of niches [8]. Another important characteristic of these bacteria is their ability to form biofilms. On blood agar plates enterococci show a very diverse hemolysis phenotype; they can be α-, β- or γ hemolytic. *Enterococcus* usually inhabits the intestinal tract of humans, but they are also isolated from the environment and are present in the gut microflora of all mammals [9].

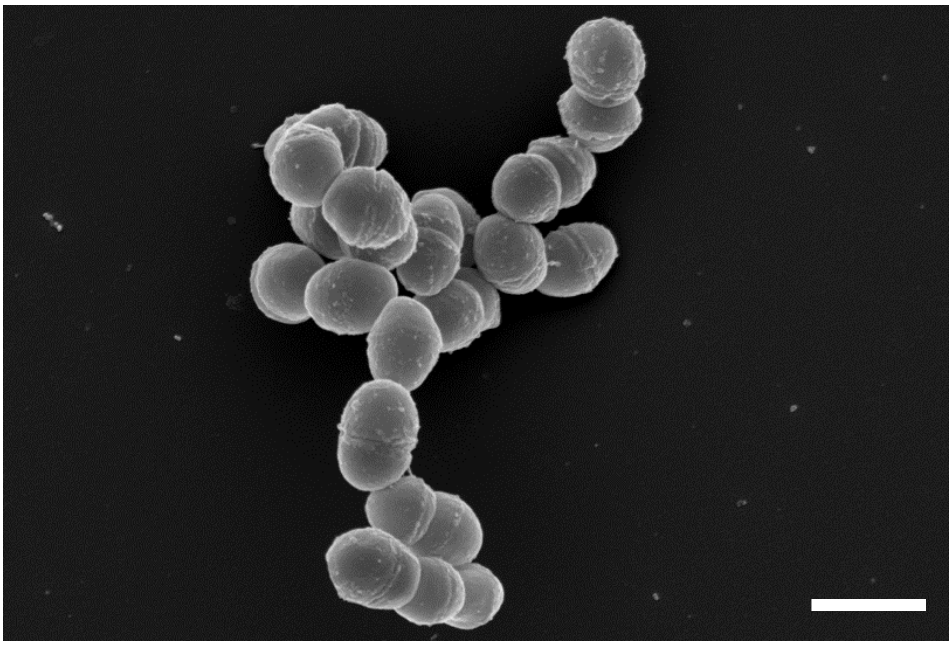

FIG. 1.1.: *Enterococcus faecalis*. Scanning electron microscopy image showing a typical enterococcal chain; bar represents 1 µm (taken by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research).

The occurrence of different enterococcal species in humans is strongly dependent on age and diet. The most frequent species of the genus *Enterococcus* in humans are *Enterococcus faecalis* and *Enterococcus faecium*, which account for more than 90% of all clinical isolates. Besides *E. faecalis* and *E. faecium* there are more than 50 other species in this genus. Other enterococci species present in humans are *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus mundtii*, and *Enterococcus raffinosus* [10].

1.2. Diseases caused by *Enterococcus faecalis*

Enterococci are part of the normal human fecal flora and widely distributed among humans [11], but also among farm animals [12]. Since the beginning of the antibiotic era, these bacteria propose major health challenges, including the need for combination antibiotic therapy to successfully treat diseases like enterococcal infective endocarditis [10]. In case of enterococcal infective endocarditis, the mortality approaches 20% [13]. Diseases caused by enterococci are reported as the third most common causes of nosocomial infections in the US

and Europe [14, 15]. About 12% of all nosocomial infections in the US and 26.5% of urinary tract infections in Germany are caused by this pathogen [16, 17].

E. faecalis has been reported to be the most common enterococcal species in hospitals and is responsible for up to 90% of infections caused by enterococci [6]. If patients are infected with *E. faecalis*, soft tissue wounds or the urinary tract is the predominant site of infection. In the beginning 1990s, surgical-site infections of patients caused by *E. faecalis* have become an increasingly important problem in clinical settings [18].

Besides urinary tract infections [19], these bacteria are responsible for intraabdominal and intrapelvic abscesses [20]. *E. faecalis* is also a serious problem for blood stream infections and sepsis [19, 21] and there are also case reports of infections of cellulitis and osteomyelitis, the respiratory tract as well as the central nervous system or meningitis [22, 23] and underlining the broad spectrum of enterococcal induced diseases.

1.3. Emergence of antibiotic resistance of Enterococcus *spp.*

Most enterococci are resistant to many antibiotics and can handle many commonly used antibiotics like aminoglycosides, aztreonam, cephalosporins, clindamycin, the semisynthetic penicillin nafcillin and oxacillin, linezoid, trimethoprim-sulfamethoxazole and quinupristin-dalfopristin [24, 25].

The broad-spectrum antibiotic resistance of enterococci is a tremendous problem in the clinical context. Especially, the increasing resistances against so-called “last resort-antibiotics” like vancomycin or teicoplanin propose a major challenge and make an effective treatment of enterococcal infections difficult. Only 15 years after the first treatment with vancomycin the first case of vancomycin-resistance was observed [15]. This resistance of enterococci is becoming more and more common with rapid accumulation of resistances to multiple antibiotic classes, for example with glycopeptides antibiotics [7, 8, 15].

Brown *et al*. showed that glycopeptide-resistant enterococci elevated the rate of mortality up to 52% in patients suffering from enterococci infections [26]. Another challenge is the high level expression of β-lactamase and aminoglycoside resistant enterococci in nosocomial diseases [27, 28]. Additionally, enterococci are also able to transfer their resistance to other unrelated Gram-positive bacteria such as *Staphylococcus aureus* [29].

Furthermore, antibiotic resistance is also an increasing problem in the food industry and intensive animal husbandry and not only limited to hospitals [30].

1.4. Virulence factors of *Enterococcus faecalis*

Some virulence factors of pathogens are regulated by virulence coding genes, present in specific regions on the genome which are termed pathogenicity islands or PAI [31]. The PAI of *Enterococcus* was first described by N. Shankar *et al*. in 2001 to be present in the genome of a multi-drug-resistant strain called *E. faecalis* [MMH594] that was collected in a cohort study in the 1980s [32]. The PAI contains multiple pathogenicity factors, *e.g.* the enterococcal surface protein (ESP), which is responsible for an increased biofilm formation and colonization potential, a cytotoxin, the aggregation substance, a bile acid hydrolase as well as other surface proteins and general stress response proteins [33].

1.4.1. Enterococcal surface protein

Enterococcal surface protein (ESP) is a cell wall-associated protein of *E. faecalis* isolates [34]. It has been demonstrated that the frequency of *esp* coding gene is more frequent in clinical isolates than commensal isolates [35]. ESP enhances the persistence of *E. faecalis* in the urinary bladder during urinary tract infections. This has been studied using isogenic ESP-deficient mutant strains in comparison to wild-type bacteria in murine infection models [36]. This study demonstrated that ESP is an important factor for adherence of enterococci to epithelial cells of the urinary bladder through specific factors such as mucin or uroplakin [36].

1.4.2. Cytolysin

Enterococcal exotoxin, also called cytolysin, is a cytotoxin that is capable to lyse erythrocytes, macrophages and neutrophils [37, 38]. The expression of the cytolysin is regulated by a two-component regulatory system via a quorum sensing mechanism [38]. Chow *et al*. described an increasing virulence potential of cytolysin-positive enterococcal strains in a rabbit model of endocarditis. In this study the authors found a positive correlation between virulence potential and cytolysin expression [39]. A retro-perspectival study showed that patients are at a five-fold higher risk to die when infected with cytolysin-positive enterococcal strains compared to infections with negative strains [32]. These findings underline the importance of enterococcal cytolysins for bacterial pathogenesis.

1.4.3. Aggregation substance

Another important virulence factor of *E. faecalis* is the pheromone-inducible surface protein aggregation substance (AS) which is essential for bacterial conjugation through promoting mating aggregate formation [40]. This strong and close association with other bacteria results in an efficient enterococcal donor-recipient plasmid transfer. It has been reported that the aggregation substance AS contributes to the pathogenesis of enterococcal infection *in vivo* through various mechanisms [41].

In addition, AS-expressing enterococci are more resistant to be killed by phagocytic cells like macrophages or neutrophils due to an inhibition of reactive oxygen species (ROS) [42].

1.4.4. Adhesin to collagen binding by *E. faecalis*

Another adhesin of *E. faecalis* is called adhesin to collagen binding by *E. faecalis* (ACE) and binds to the extracellular matrix [43]. ACE is a microbial surface component recognizing adhesive matrix molecule (MSCRAMM) on the enterococcal surface and an important virulence factor contributing to virulence in urinary tract infection models *in vivo* [44].

The function and structure is similar to the staphylococcal collagen binding protein CNA [45]. ACE is present in nearly 100% of clinical enterococcal isolates, suggesting that ACE is expressed *in vivo* and contributes to virulence, but not necessary to cause an infection such as described in an urinary tract infection [44, 46].

1.4.5. Gelatinase

Another important virulence factor from *Enterococcus faecalis* is an extracellular active gelatinase (GelE). This extracellular zinc-metalloprotease is part of a protection mechanism of *E. faecalis* against the host response [47]. GelE is important for resistance to several key components of the host innate immune defense system including antimicrobial peptides preventing damage to the pathogen [48]. The functional spectrum of GelE includes cleavage and degradation of host proteins like LL-37, fibrinogen, fibrin, collage, endothelin-1 and bradykinin. Degradation of components of the complement system like C3, C3a and C3b results in an inactivation of the complement cascade [47, 49-51]. Previous studies showed that GelE-positive strains of *Enterococcus faecalis* have an increasing potential to establish a biofilm [52]. Furthermore, expression of GelE increased the virulence in an animal model of

infective-endocarditis [52, 53] as well as in an invertebrate model of *E. faecalis* infection [54]. These findings underline the importance of GelE for host colonization and virulence.

1.5. Interaction of *E. faecalis* with the host immune system

Based on the clinical importance of *E. faecalis* infections it is surprising that the knowledge about the interaction of enterococci with the host immune system is very limited and fragmentary.

Most studies focus on the interaction of *E. faecalis* with phagocytes like macrophages and polymorphonuclear neutrophils (PMNs). A few studies have shown that opsonization of *E. faecalis* and subsequent killing by PMNs plays a crucial role in the host immune response to enterococci [55]. Also the translocation of *E. faecalis* in mice, which could lead to sepsis, is depending on functionally active PMNs and macrophages [56]. An AraC-type transcriptional regulator called PerA seems to be necessary for *E. faecalis* to survive intracellularly in phagocytes like macrophages [57]. Also Zou *et al.* showed that *E. faecalis* inhibits the activation of lysosomes by an unknown mechanism, enabling *E. faecalis* to survive within these cells [58]. PerA itself is known to be able to bind to human platelets and seems to be also involved to biofilms formation with other substance [59, 60].

On the host site it was postulated that the TLR/MyD88 signaling pathway plays an important role for infection outcome of infections caused by this microorganism [61, 62].

1.6. Recognition of pathogens by pattern recognition receptors (PRRs) on host immune cells

To identify non-host related structures eukaryotic cells are expressing pattern recognition receptors (PRRs). These PRRs are an important part of the immune system because they allow host immune cells to identify other cells or structures, like microbial agents or pathogen-associated molecular patterns (PAMPs), which are conserved structures expressed on their surface [63, 64].

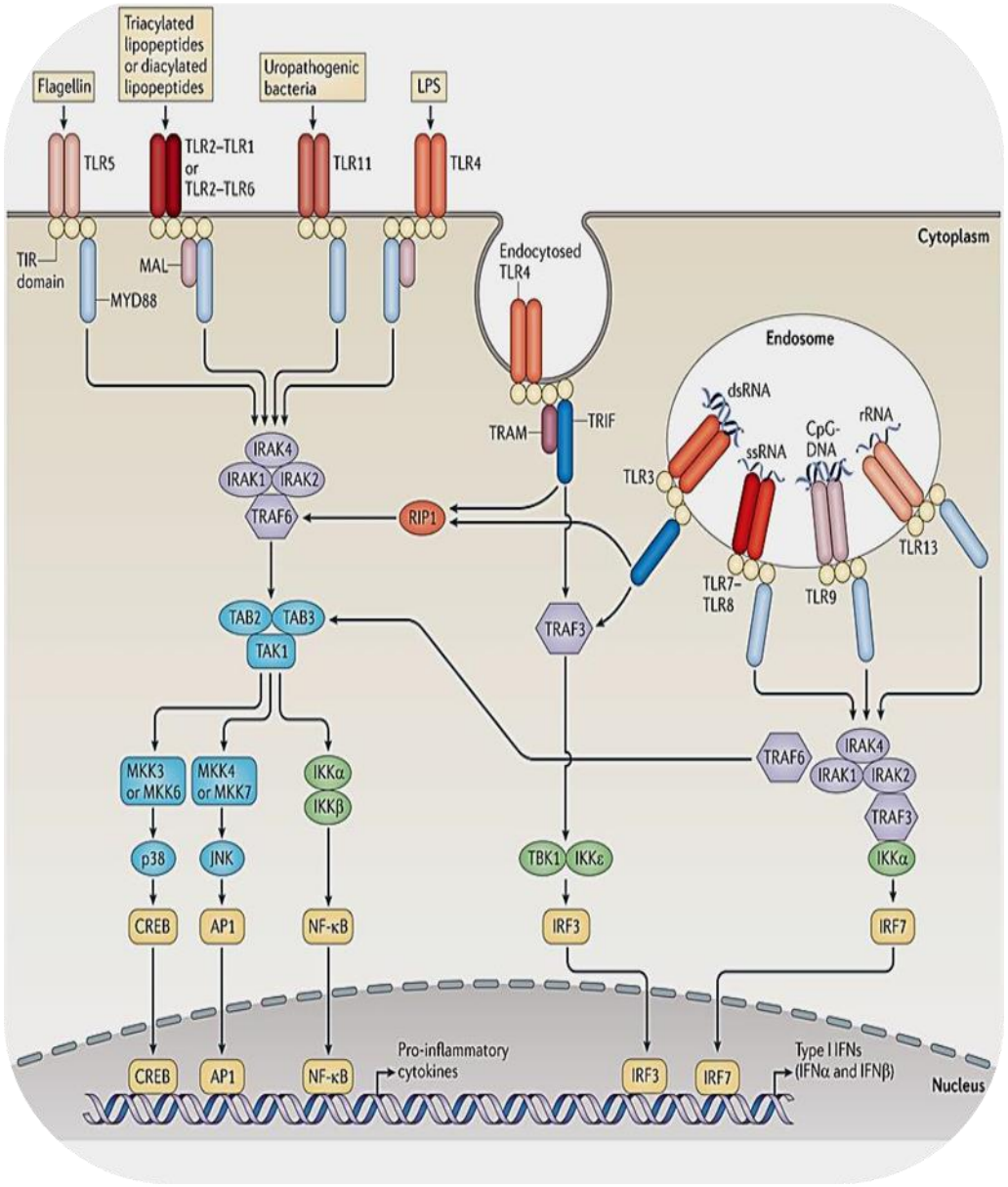

FIG. 1.2.: TLR signaling pathway. A detailed but simplified figure of the TLR signaling pathways modified from O'Neill, Golenbock [65].

PRRs also identify damage-associated molecules pattern (DAMPs) [66]. Mammals have evolved many different groups of PRRs, including AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), Toll-like receptors (TLRs) and intracellular DNA sensors such as cGAS [67]. TLRs are one of the best-characterized PRRs. They are not only responsible for sensing invading pathogens outside the cell but also intracellularly within endosomes and lysosomes [68]. The number of TLRs varies from species to species and estimates range from 10-13. In humans ten functional TLRs have been identified so far and named TLR1-10. Furthermore a nonfunctional pseudogene

called TLR11 has been described. In mice 12 functional TLRs have been reported, named TLR1-9 and TLR11-13 [69]. Different TLRs recognize different molecular patterns of microorganisms and self-components [66, 70, 71].

Table 1. Overview of TLRs in mammals

Receptor	**Location**	**Ligand**	**Adaptor molecule**
TLR2/1	Cell surface	triacylated lipoprotein, lipopeptide	MyD88/TIRAP
TLR2/6	Cell surface	LTA from Gram-positive bacteria, PGN, diacylated lipoprotein, lipopeptide,	MyD88/TIRAP
TLR3/3	intracellular	viral dsRNA	TICAM1
TLR4/4	Cell surface / intracellular	LPS from Gram-negative bacteria, HSP72, S100A8/A9, MBD2	MyD88/TIRAP TICAM1/TICAM 2
TLR5/5	Cell surface	bacterial flagellin	MyD88
TLR7/7	Intracellular	viral ssRNA	MyD88
TLR8/8	Intracellular	viral ssRNA	MyD88
TLR9/9	Intracellular	bacterial or viral CpG-DNA	MyD88
TLR10/1,2,10 (human specific)	Cell surface	ND	MyD88
TLR11/11 (mouse specific)	Intracellular	*Toxoplasma gondii* profiling, uropathogenic bacteria	MyD88
TLR12/12,11 (mouse specific)	Intracellular	DNA from *Toxoplasma gondii*	MyD88
TLR13 (mouse specific)	Intracellular	23S rRNA, vesicular stomatitis virus	MyD88

PGN: peptidoglycan; LPS: lipopolysaccharide; LTA: lipoteichoic acid; HSP: heat shock protein; MBD2: murine β-defensin; CpG-DNA: cytosine-phosphate-guanine DNA; MyD88: myeloid differentiation primary response gene 88; TIRAP: TIR-domain-containing adaptor protein; TICAM: TIR-domain-containing adaptor molecule; ND: not determined [72].

1.6.1. Function of Toll-like receptor 2 (TLR2)

Toll-like receptor 2 (TLR2) is one of the most prominent receptors in the recognition of PAMPs of Gram-positive bacteria like lipoteichoic acid (LTA) or peptidoglycan, which composes the Gram-positive cell wall [73, 74].

TLR2 is expressed on the surface of many different immune cells as well as endothelial cells and belongs together with TLR1, TLR5, TLR6, TLR10, and TLR11 to the class of cell surface TLRs [66, 75, 76].

Additionally, TLR2 recognizes also non-microbial ligands, including DAMPs such as high mobility group 1 protein (HMGB1) or hyaluronan fragments and other products of inflamed tissue [77-80].

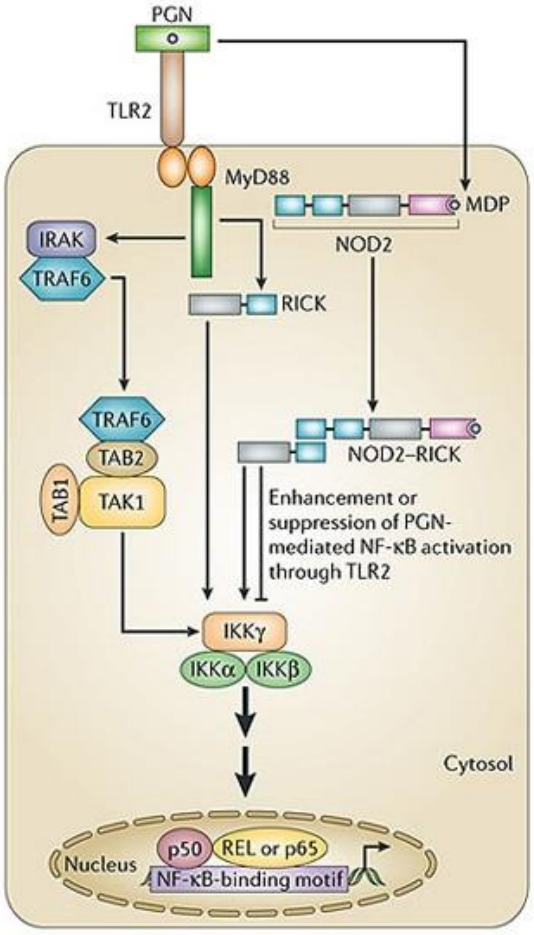

FIG. 1.3.: TLR2 signaling pathway. TLR2, together with other TLRs, shares the MyD88-dependent pathway, which finally activates NF-κB, leading to the production of inflammatory cytokines like IL-6 and IRF3. Adapted and modified from [81].

TLR2 and TLR4, the latter one being the major receptor for the recognition of Gram-negative bacteria, initiates intracellular signaling pathways that lead to activation of transcription factors, such as nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3). This activation results in the transcription of genes that are part of immune regulation including proinflammatory cytokines, like IL-6, IFN-γ and TNF-α (Fig 1.3) [78].

1.7. Cells of the innate immune system and their roles in bacterial infections

Innate immunity is the first line of defense against invading microbial pathogens and, therefore, the first point of contact. The innate immune defense is an essential first step in controlling infectious disease. To establish infections, pathogens evolved strategies to overcome this defense. Innate immunity consists of the humoral components such as the complement system and also cellular components including PMNs, macrophages, mast cells, basophiles, eosinophils and dendritic cells. Resistance mechanisms against the innate immune system that enable this commensal organism to become pathogenic are widely unknown. Some of the key components of the host innate immune system as well as their role in *E. faecalis* induced infections are described in detail below.

1.7.1. Biology of mast cells

Mast cells (MCs) are multifunctional and highly effective tissue dwelling cells and an important component of the immune system [82]. MCs develop from hematopoietic progenitor cells. They circulate in an immature form in the blood stream and undergo a final differentiation step by migrating to vascularized tissues where they maturate in the presence of stem-cell factors and other cytokines like IL-3 to fully functional cells [83]. Maturated MCs are commonly found at sites in the body that are exposed to the external environment such as the skin, the lung or the intestinal tract, making these cells important and effective sentinels for the immune system to recognize invading microbial pathogens [84]. They also are found in close proximity to blood vessels, where they can efficiently regulate vascular permeability [85].

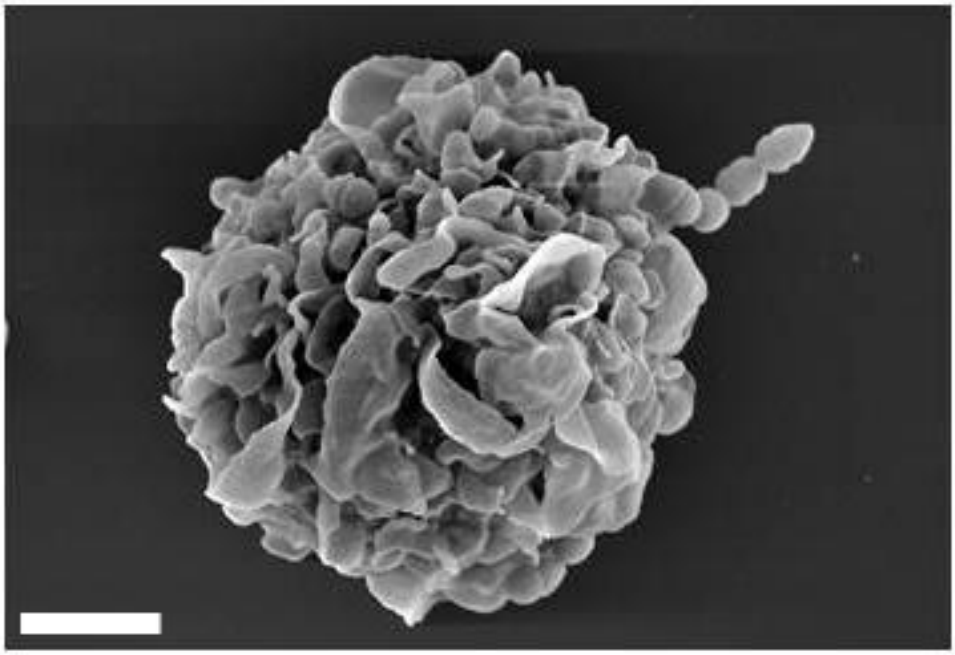

FIG. 1.4.: Scanning electron microscopy of a bone marrow mast cell (taken by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research). Bar: 2µm.

Even though they do not have direct cell-cell contact with antigen-presenting cells, MCs can modulate the behavior and recruitment of other neighboring effector cells through the release of immunological active mediators [86]. There are four main classes of mediators released by MCs, which are released in a process called degranulation. The four classes are 1st cytokines and 2nd chemokines, which are produced and pre-stored in granular compartments. The 3rd class are newly generated lipid-mediators and 4th are endogenous antimicrobial agents such as antimicrobial peptides or reactive oxygen species (ROS) [87].

These components can mediate a variety of antimicrobial activities such as the coordination of immune cell recruitment to the site of infection [87] or the release of antimicrobial peptides like the cathelicidin LL-37 [88].

Table 2. Mediators released by mast cells

Mediators released by mast cells and their function activity		
Granule associated	Histamine, serotonin	Alteration of vascular permeability
	Different proteases	Remodel tissues and recruit effector cells
Lipid-derived	LTC_4, LTB_4, PGD_2, PGE_2	Recruit effector cells, regulate immune

Mediators released by mast cells and their function activity		
		response, promote angiogenesis
Cytokines	TNF-α, IL-3, IL-6, IFN	Induce inflammation
	IL-10, TGF, VEGF	Regulate inflammation and angiogenesis
Chemokines	CCL 2, 3, 4, 5, 11, 20	Recruit effector cells and regulate immune responses
	CXCL 1, 2, 3, 8, 9, 10, 11	Recruit effector cells and regulate immune responses
Others	Nitric oxide and superoxide radicals	bactericidal
	Antimicrobial peptides like LL-37	bactericidal

Furthermore, several studies have shown that MCs are able to phagocytize numerous Gram-negative bacteria [89] and that they are also able to kill several Gram-positive bacteria by extracellular mechanisms like *Streptococcus pyogenes* [88] or *Staphylococcus aureus* [90].
For a long time, MCs were only seen as part of allergic reactions and their activation during parasitic infections, but not as a benefit for the host in bacterial, viral or fungal infections [86, 91, 92]. However, MCs are more and more recognized as a crucial part in the response to bacterial infections due to the release of pre-stored and newly synthesized inflammatory mediators as well as antimicrobial components [93].

1.7.2. Response of mast cells to microbial pathogens

Besides their known role in the initiation of allergic reactions, chronic inflammatory processes and activation during certain types of parasitic infections [91, 92], no clear evidence demonstrates that MCs also play a prominent role in the early immune response to invading pathogens [93, 94].
MCs employ many different mechanisms in response to invading microbial pathogens. MCs are able to recognize opsonized bacteria and phagocytize them. Sher and his colleagues were one of the first researchers who studied the phagocytotic behavior of MCs in the late 1970s with heat-killed *Salmonella enterica* serovar Typhimurium [95]. They could show that heat-killed *S. typhimurium* incubated in rat serum lead to an opsonization with iC3b which is

recognized through the complement receptor 3 of MCs. This recognition in turn leads to an activation of MCs.

Malaviya *et al.* showed phagocytic activity of MCs with other enterobacteriaceae such as *Enterobacter cloacae, Escherichia coli* and *Klebsiella pneumoniae* [96].

In addition to the intracellular activity, MCs have also an extracellular killing mechanism. Von Köckritz-Blickwede showed that MCs release extracellular trap that are similar to neutrophil extracellular traps (NETs) to trap and ensnare bacteria [97]. These structures were named mast cell extracellular traps (MCETs).

MCs contain a lot of antimicrobial peptides, which are released upon infection. One of these components is the cathelicidin LL-37 [88, 98]. The full function of this antimicrobial peptide on bacterial surfaces is not fully understood yet, but treatment with LL-37 resulted in a surface deformation of Gram-positive bacteria like *S. pyogenes* [99].

Beside these direct interactions with bacteria, MCs are also important immunomodulatory cells. They are involved in the recruitment of other immune cells like neutrophils by the release of a broad-spectrum proinflammatory cytokines including TNF-α or IL-6 *in vitro* and *in vivo* [100-102]. Nevertheless, the role of these innate immune cells in the course of many bacterial infections is still poorly understood and requires more and intensive research.

1.7.3. Biology of dendritic cells

The immune system is divided into the innate immune system and the adaptive immune branch coordinated by specific immune cells of which dendritic cells (DCs) are of central importance.

DCs are able to sense microbial pathogens as well as microbial products and initiate an effective immune response against pathogens [103].

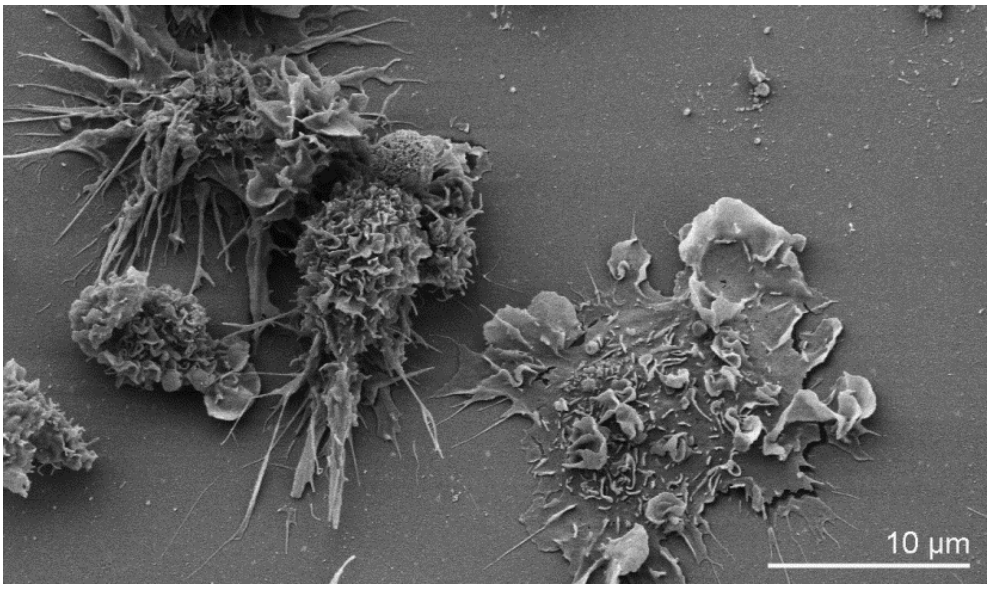

FIG. 1.5.: Scanning electron microscopy of immature dendritic cells in the bone-marrow (taken by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research). Bar: 10µm

The maturation status of DCs is in particular characterized by the production of the inflammatory cytokines interleukin 12 (IL-12) as well as by the upregulation of the costimulatory molecules CD40, CD80 & CD86 [104, 105]. These markers are often used to discriminate functional DCs, which are important for priming T-cells [106].

Just like many antigen presenting cells DCs are able to release IL-12 during infection *in vitro* and *in vivo.* IL-12 is an important cytokine, which is crucial to trigger the differentiation of naive $CD4^+$ T-cells into T-helper 1 cells (T_H-1) [105, 107]. In response to pathogens, DCs are important in infections caused by the human pathogens *S. pyogenes, S. aureus* or *Listeria monocytogenes* by inducing a protective immune response [107-109]. Despite the important function as a sentinel cell [105] and the initiation and mounting of an effective innate immune response against several pathogenic bacteria, the knowledge of the function and role of these cells in the cause of *E. faecalis* infections is still incomplete.

1.7.4. The role of dendritic cells during microbial induced infections

Dendritic cells are antigen-presenting cells (APCs). To present antigens on their surface, these cells phagocytize and process antigens from their environment. In contrast to other phagocytic active antigen-presenting cells like neutrophils or macrophages, which are strongly involved in the elimination of invading microorganisms, the main function of DCs is to preserve and present antigen information [110]. Antigens are loaded intracellularly on the MHC-II complex and transported to the surface where they are presented to T-cells [111].

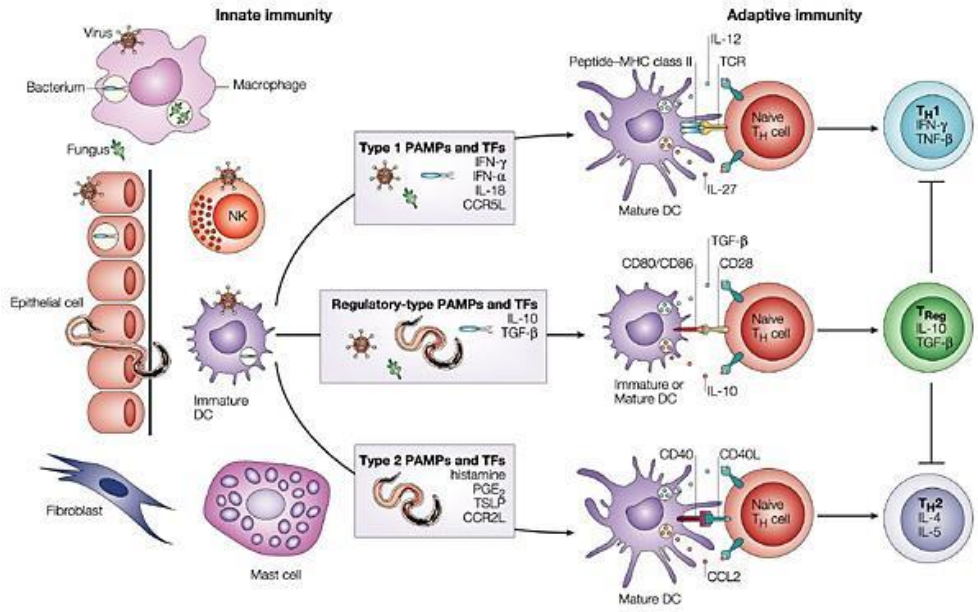

FIG. 1.6.: Maturation of DCs. The maturation of dendritic cells is triggered by many different factors, generated by other immune cells like macrophages, epithelial cells, fibroblast and mast cells when these cells encounter microbial organisms or agents and become activated. They produce type 1, type 2 or regulatory-type PAMPs and tissue factors (TF) which leads to different stages of matured DCs which in turn prime naïve $CD4^+$ T-cells into T_H-1, T_H-2 or regulatory T-cells (T_{REG}-cells) [112].

This mechanism is very important to mount an effective immune response and to induce the differentiation and proliferation of cells of the adaptive immune system like T_H-1 cells [106]. However, DCs are capable to elicit a strong killing ability, for example in the immune response against *Streptococcus pyogenes* [113].

Recognition of microbes by DCs is mediated through different PRRs such as TLRs [67, 114]. TLRs are important members of the IL-1 receptor/TLR superfamily. TLRs recognize the presence of microbial pathogens via the detection of conserved microbial structures called PAMPs [67]. The maturation of DCs can directly be initiated by the activation of TLRs [114]. While most TLRs act through MyD88 [115], some of them like TLR4 recognizing LPS induce maturation in a MyD88 independent manner [114].

The interactions of TLRs with their target motifs as well as the intracellular signaling cascades are discussed more into detail in section 1.6. and are mostly depending on the adapter molecule MyD88. Studies using MyD88 knockout systems have shown the importance of this pathway for the activation and the maturation of fully functional DCs [116-118].

DCs also mature indirectly and independent of pathogenic stimuli by sensing a large number of inflammatory-associated factors like *e.g.* MIP-1α and MIP-1β from infected endothelial cells [112, 119].

Once DCs are maturated, they migrate to the lymphatic system. Here they present the antigens loaded onto class II MHC to T-cells and prime them into different classes like T_H-1, T_H-2 or T_{REG}-cells [120], an important step in the initiation of an adaptive immune response.

1.8. Endothelial cells as part of the immune system

1.8.1. Biology of endothelial cells

As an essential source for cytokine production, the endothelium is an important part of the host innate immune response. It also is the natural barrier covering the interior blood- and lymphatic vessel system [121]. Endothelial cells (ECs) grow in a confluent monolayer and appear as cobblestone. This form can be changed through culturing them under shear stress so they elongate in flow direction [122, 123].

Endothelial cells (ECs) and cells of the hematopoiesis have a common origin. Cells with *tek*-expression – a receptor tyrosine kinase – seems to be linked to the EC linage while *c-myo* expression is linked to the hematopoietic cell differentiation [124].

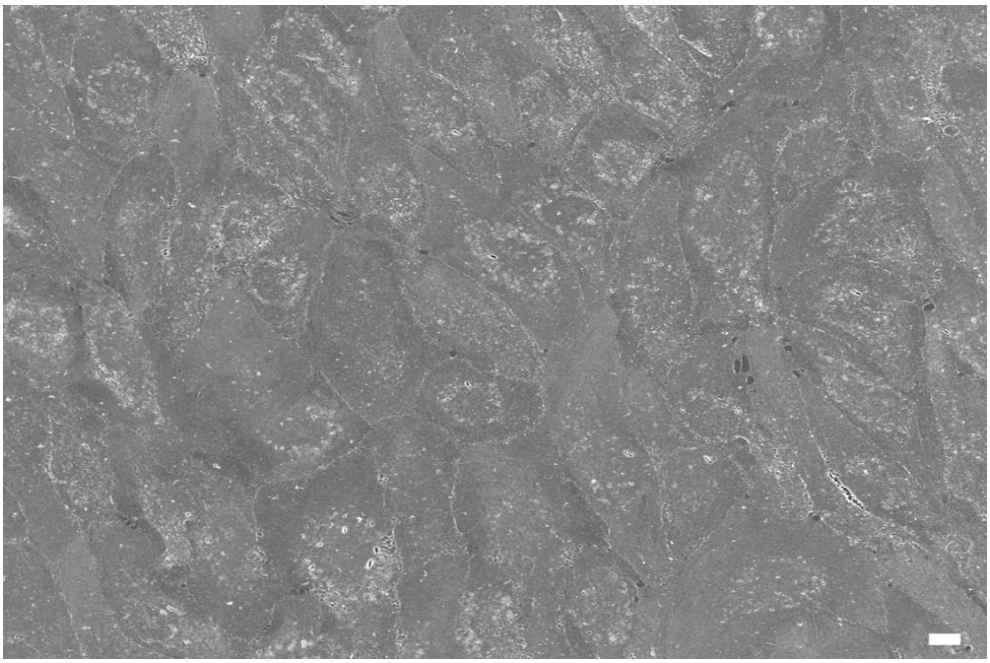

FIG. 1.7.: Scanning electron microscopy of a confluent monolayer of human umbilical vein endothelial cells cultured for 120h under flow conditions (taken by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research). Bar: 20µm

ECs are characterized by different molecules presented on their surface like platelet-endothelial cell adhesion molecule 1 (PECAM-1 or CD31), CD34, glycoprotein Ia, IIa, IIIa and P-selectin (CD62P) and in addition by intracellular factors like platelet-derived growth factor, transforming growth factor-β and von-Willebrand-factor which are pre-stored in so called Weibel-Palade bodies [125, 126]. ECs are also identified by the uptake of acetylated low-density lipoprotein (LDL) and the expression of factor 8 [127].

Other important surface molecules expressed on ECs are intercellular adhesion molecule 1 (ICAM-1 or CD54), E-selectin (CD62-E), vascular cell adhesion protein 1 (VCAM-1 or CD106) and mucosal addressin cell adhesion molecule 1 (MA*d*CAM-1) which are necessary for the migration process of leukocytes over the endothelial layer [128].

ECs produce a broad spectrum of different factors, which are necessary for the regulation of blood pressure, flow rate, angiogenesis, coagulation, growth regulation and production of extracellular matrix proteins [125, 129]. ECs also release important components, *e.g.* IL-6 and IL-8, which are responsible for inflammatory immune processes like neutrophil recruitment and activation [130-133].

1.8.2. Anti-pathogenic functions of endothelial cells

Endothelial cells are autocrine for cytokines and produce a broad spectrum of pro- and anti-inflammatory cytokines (Table 3.) [134-137]. Besides to the recruitment of immune cells like PMNs the endothelium is involved in a process called “rolling” of leukocytes involving cells like PMNs. This rolling process is based on the expression of a small number of surface proteins on ECs. In the initial step rolling involves the binding of PMNs to E- and P-selectin presented on the surface of ECs. This leads to an enhanced exposure of PMNs to cytokines and chemokines because of the release by the endothelium [138, 139]. In a second step, the leukocyte rolling process starts. Integrins are expressed on the surface of PMNs, which increases the adhesion between PMNs and ECs [138, 140]. The next step is characterized by the migration of PMNs along the endothelium. In this step, the connection to the EC is based on the interaction of LFA-1/ICAM-1, VLA-4/VCAM-1 and a4b/MA*d*CAM-1. The last step in the rolling process of PMNs is the transmigration into the sub-endothelium space. The initiation of transmigration is ICAM-1 depended [141] while the actual step is characterized by weakening the tight junction between single endothelial cells. The molecular basis of this process is still not fully understood, but there are indications that an intracellular increase of Ca^{2+} leads to an activation of p38 and a signaling via ICAM-1 binding in turn results in an activation of RhoA and both enhance the potential of PMN transmigration [142-144]. The expression of adhesion molecules on the surface of ECs enables PMNs to reach the site of infection and to combat invading microbial pathogens.

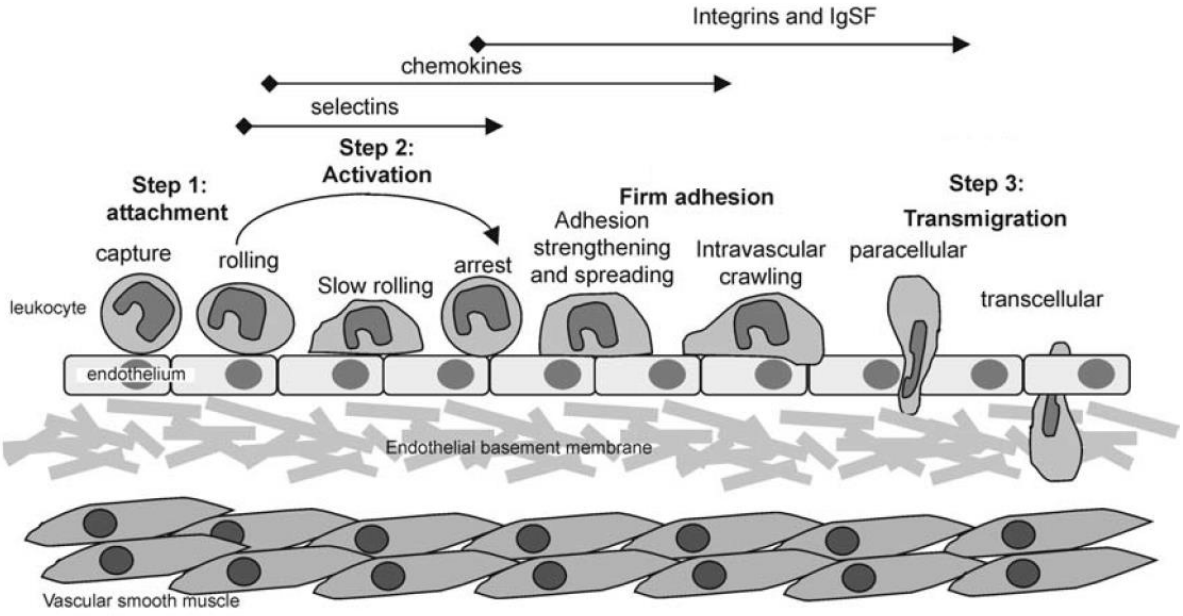

FIG 1.8.: Simplified model of the rolling process of leukocytes along the endothelium. The process is divided in three steps: (1) attachment – leukocytes binds to surface presented E- and P-selectin; (2) activation – the binding become stronger by expression of integrins on EC surface and leukocytes start migration over EC; (3) transmigration – leukocytes go deeper into sub-endothelial space. Modified from Lawson *et al.* [128]

ECs have a direct antimicrobial effect against pathogens by the release of antimicrobial peptides like human β-defensin 3 [145] and RNase7 [145, 146].

Additionally, ECs are able to modify their permeability and stiffness. This process is regulated by LL-37. Byfield and colleagues showed a concentration-dependent increase in stiffness by LL-37 whereas at the same time the permeability was reduced [147]. These modifications could protect the host from spreading bacteria like *Streptococcus pyogenes*, *Corynebacterium diphtheriae* or *Leptospira interrogans* [148-150].

Table 3. Released components by endothelia cells

Mediator released by endothelia cells	
Cytokine	IL-1; IL-3; IL-6; IL-8; IL-11; IL-15; TGF-β; TNF-α
Chemokines	CXCL-1; CCL-2
Other released components	GM-CSF

1.9. Aim of this study

The aim of this study was to enhance the understanding of molecular and cellular factors and processes involved in host responses to infections caused by the nosocomial pathogen *E. faecalis*. This pathogen rapidly becomes an increasing threat in ICUs and is of rising importance as a reason for nosocomial infections and is producing as a result increasing costs in the health care system.

A major focus of this study is how cells of the innate immune system respond to infections caused by this pathogen and which resistance mechanisms and strategies *E. faecalis* employs to circumvent these host defense strategies. This interplay is of particular significance due to the shift from a commensal to a pathogenic organism.

Because studies in particular in terms of enterococcal interactions with host innate immune cells, like mast cells (MCs), dendritic cells (DCs) or the endothelial barrier are insufficient so far the central interest of this study was to gain deeper insights into these interactions.

Another part of this thesis focused on the receptors and signaling pathways involved in response to this microorganism, in particular the role of PRRs in the recognition of enterococci by host immune cells.

The results of this study contributes to a better understanding of the interactions of *E. faecalis* with the cellular compartment of the host innate immune system as well as to gain further insights into the pathogenicity of *E. faecalis* infections and the role of cells of the innate immune response in the course, development and resolution of *E. faecalis* induced diseases.

2. Materials and Methods

2.1. Chemicals

Agarose	ROTH
Ammonium chloride	MERCK
Alexa-Fluor 488 Phalloidin	THERMO-FISHER
BEA (bile esculin agar)	BROTH
β-mercaptoethanol	MERCK
BSA (bovine serum albumin)	SIGMA
BHI (brain heart infusion)	ROTH
Cacodylate	ROTH
Calcium chloride	ROTH
Carboxyfluorescein	SIGMA
Comp 48/80	SIGMA
Cromolyn	SIGMA
Cytocalasin D	SIGMA
DAPI (4′,6-Diamidin-2-phenylindol)	SIGMA
Disodium hydrogen phosphate	MERCK
DTNB	SIGMA
EDTA	GIBCO
Ethanol	LIV
Glucose	ROTH
Glutaraldehyde	ROTH
Glycerol	MP
Hematoxylin	MERCK
Heparin	ROCHE
HEPES	GIBCO
Isopropanol	LIV
L-Glutamine	GIBCO
Magnesium chloride	ROTH
N-carbobenzoxy-lysine thiobenzyl ester	SIGMA
OptiPrep	AXIS-SHIELD
PBS (phosphate buffered saline)	SIGMA
PFA (paraformaldehyde)	FLUCA
PNAG (p-nitrophenyl N-acetyl-β-D-glucosamide)	SIGMA

Poly-l-lysine	SIGMA
Potassium chloride	MERCK
Potassium dihydrogen phosphate	MERCK
Potassium hydrogen carbonate	MERCK
ProLong Slowfade + DAPI	INVITROGEN
RLT-Buffer	QIAGEN
RNase free water	GIBCO
Saponin	SIGMA
Sodium azide	SIGMA
Sodium dihydrogen carbonate	MERCK
Sodium chloride	MERCK
Sulfuric acid	ROTH
TMB	MERCK
TPA	MERCK
Toluidine-Blue	ROTH
TRIS	ROTH
Triton X-100	SIGMA
Trypan blue	SIGMA
Tween20	ROTH
Yeast extracts	BECTON, DICKINSON

2.2. Devices and consumables

2.2.1. Devices

Agarosegel-electrophoresis chamber	GIBCO
Autoclave	TECNOMARA
Centrifuges:	
-5417 R	EPPENDORF
-MEGAFUGE 1.0	HERAEUS
Clean bench	THERMOFISHER
Cuvette	ROTH
ELISA-Reader Sunrise	TECAN
Flow-Cytometry Units:	
-FACSCalibur	BECTON DICKINSON
-LSR II	BECTON DICKINSON

Frezzer (-20°C & -80°C)	LIEBHERR
Fridge	LIEBHERR
Heatblock	EPPENDORF
Horizontal	SARTORIUS
Incubators:	
- CO_2 Incubator	HERAUES
- Scientific water mantled incubator 3111	FORMA
Microscope:	
-EM Microscope DSM982 Gemini	ZEISS
-EM 910 Transmission-electron-microscope	ZEISS
-Axiophot microscope with an attached Axiocam HRc digital camera	ZEISS
Microwave	BROTHER
PCR-Cycler	EPPENDORF
pH-Meter	SCHOTT
Photometer Novaspec II	PHARMACIA LKB
Pipette (10 µL; 20 µL; 100 µL; 200 µL; 1000 µL)	EPPENDORF
Vortex mixer	HOBEIN
Water bath (heated)	KÖTTERMAN

2.2.2. Consumables

96-well micro-titer plates	SARSTEDT
Cannula	BRAUN
Cell culture plates (4, 6, 24, 48 wells)	SARSTEDT
Cell strainer	SIGMA
Falcon Tube (15 ml; 50 ml)	SARSTEDT
Gloves (latex; nitrile)	SHIELD-SCIENTIFIC
Microtiter plate	NUNC
Petri dish	SARSTEDT
Pipette tips	EPPENDORF
Reaction vessels (0.2 µL; 1.5 ml; 2 ml)	EPPENDORF
Syringe (1 ml; 5 ml; 20 ml)	BRAUN

2.3. Antibodies, enzymes and sera

2.3.1 anti-human

anti-human CD54 *Pacific Blue*	BIOLEGEND
anti-human CD54, purified	BIOLEGEND
anti-human CD62E APC	BIOLEGEND
anti-human CD62E, purified	BIOLEGEND
anti-human CD106 PE	BIOLEGEND
anti-human CD106, purified	BIOLEGEND
anti-human IL-1β, purified	BIOLEGEND
anti-human IL-1β, biotinylated	BIOLEGEND
anti-human IL-6, purified	BIOLEGEND
anti-human IL-6, biotinylated	BIOLEGEND
anti-human IL-8, purified	BIOLEGEND
anti-human IL-8, biotinylated	BIOLEGEND
anti-human MCP-1, purified	BIOLEGEND
anti-human MCP-1, biotinylated	BIOLEGEND
anti-human TNF-α, purified	BIOLEGEND
anti-human TNF-α, biotinylated	BIOLEGEND

2.3.2 anti-mouse

anti-mouse CD11b PE/Cy7	BIOLEGEND
anti-mouse CD11b PE	BIOLEGEND
anti-mouse CD11c *Alexa Flour 647*	BIOLEGEND
anti-mouse CD11c FITC	BIOLEGEND
anti-mouse CD11c PE	BD PHARMINGEN
anti-mouse CD16/CD32	BD PHARMINGEN
anti-mouse CD29 *Alexa Flour 488*	MILTENY BIOTEC
anti-mouse CD40 *Alexa Flour 488*	BIOLEGEND
anti-mouse CD80 PE/Cy5	BD PHARMINGEN
anti-mouse CD86 PE/Cy7	BIOLEGEND
anti-mouse CD117-PE	BD PHARMINGEN
anti-mouse F4/80 FITC	BIOLEGEND
anti-mouse F4/80 PE	BIOLEGEND
anti-mouse IL-6, purified	BIOLEGEND

anti-mouse IL-6, biotinylated	BIOLEGEND
anti-mouse IL-10, purified	BIOLEGEND
anti-mouse IL-10, biotinylated	BIOLEGEND
anti-mouse IL-12 p70, purified	BIOLEGEND
anti-mouse IL-12 p70, biotinylated	BIOLEGEND
anti-mouse IFN-γ, purified	BD OptEIA™
anti-mouse IFN-γ, biotinylated	BD OptEIA™
anti-mouse KC, purified	BD PHARMINGEN
anti-mouse KC, biotinylated	BD PHARMINGEN
anti-mouse Ly6G / Ly6C *Alexa Flour 488*	INVITROGEN
anti-mouse Ly6C PE	INVITROGEN
anti-mouse MIP2, purified	BD PHARMINGEN
anti-mouse MIP2, biotinylated	BD PHARMINGEN
anti-mouse TNF-α, purified	BD OptEIA™
anti-mouse TNF-α, biotinylated	BD OptEIA™
anti-mouse TLR2 *Alexa Flour 488*	BIOLEGEND

2.3.3 Sera, fluorescence antibody and other enzymes

Bovine serum albumin	ROTH
Goat anti-rabbit IgG *Alexa Flour 488*	INVITROGEN
Goat anti-rabbit IgG *Alexa Flour 568*	INVITROGEN
Goat anti-mouse IgG1	BIOLEGEND
Rabbit anti-*E. faecalis* Serum	PINEDA
Streptavidin-HRP	BD OptEIA™
Fetal Calf Serum	GIBCO

2.4. Primers and Probes

Table 4. Primer-list human

Name	Forward	Reverse	Sequence	Annealing Temperature [°C]
CD54	X		5´-TGG TAG CAG CCG CAG TCA TA-3´	60
		X	5´-CTC CTT CCT CTT GGC TTA GT-3´	60
CD62E	X		5´-AAC TTC CAT GAG GCC AAA CG-3´	54
		X	5´-TTG TCG TTG CCA GTG TTC AG-3´	54
CD106	X		5´-CCC TTG ACC GGC TGG AGA TT-3´	60
		X	5´-TGG GGG CAA CAT TGA CAT AAA GTG-3´	60
β-*actin*	X		5´-TGG AAT CCT GTG GCA TCC ATG AAA C-3´	52-60
		X	5´-TAA AAC GCA GCT CAG TAA CAG TCC G-3´	52-60

Table 5. Primer-list *Enterococcus spp*.

Name	Forward	Reverse	Sequence	Reference
cylA	X		5´-GGT TAT GCA TCA GAT CTC TCA A-3´	[151]
		X	5´-CTG TAT ATA ATC TAC TTT TTC AGA AGA TAA TTC-3´	
cylL$_L$	X		5´-CTG TTG CGG CGA CAG CT-3´	[151]
		X	5´-CCA CCA ACC CAG CCA CAA-3´	

Name	Forward	Reverse	Sequence	Reference
gelE	X		5´-CGG AAC ATA CTG CCG GTT TAG A-3´	[151]
		X	5´-TGG ATT AGA TGC ACC CGA AAT-3´	
esp	X		5´-GGA ACG CCT TGG TAT GCT AAC-3´	[151]
		X	5´-GCC ACT TTA TCA GCC TGA ACC-3´	
ace	X		5´-CGG CGA CTC AAC GTT TGA C-3´	[151]
		X	5´-TCC AGC CAA ATC GCC TAC TT-3´	
asa	X		5´-GAT ACA AAG CCA ATG TCG TTC CT-3´	[151]
		X	5´-TAA AGA GTC GCC ACG TTT CAC A-3´	
ebpA	X		5´- CAA CAA CAC CAG GGC TTT TTG-3´	[152]
		X	5´-ACC GGA CCA GTC AAC GAC TAA G-3´	
ebpB	X		5´-CGT ACA GGC GGC AAG TCT TT-3´	[152]
		X	5´-AGG TAT TCC CCC GCT TGA TTT-3´	
ebpC	X		5´-GCG GCA CAC TAA AAT TCG TTT A-3´	[152]
		X	5`-GTC GTC GGT ATG ACC GTT ATC A-3´	
hyl	X		5´-ACA GAA GAG CTG CAG GAA ATG-3´	[153]
		X	5´-GAC TGA CGT CCA AGT TTC CAA-3´	

2.5. Cell culture and nutrient solutions

All buffers were stored at room temperature unless otherwise stated.

2.5.1.1. Iscove's Modified Dulbecco's Media (IMDM)

Iscove's medium was supplemented with 10% (v/v) heat-inactivated FCS (30 minutes, 65°C), 2 mM L-glutamine, 10 mM HEPES buffer, 5% (v/v) NEAA and 100 µg/l of penicillin/streptomycin.

2.5.1.2. Dulbecco's Modified Eagle's Medium

Dulbecco's medium was supplemented with 5% (v/v) heat-inactivated FCS (30 minutes, 65°C), 2 mM L-glutamine and 10 mM HEPES buffer.

2.5.1.3. Endothelia Cell Medium for cultivation

Endothelia cell medium was supplemented with 10% (v/v) heat-inactivated FCS (30 minutes, 65°C) and manufacture typical BulletKit™ was added for cultivation of all HUVECs.

2.5.1.4. Endothelia Growth Medium-1 (EBM-1)

Endothelia growth medium-1 was supplemented with 10% (v/v) heat-inactivated FCS (30 minutes, 65°C). Additionally, it BulletKit™ was added for cultivation of HUVECs from ScienCell then called ECM. In case of HUVECs from Lonza called EGM-1

2.5.1.5. Endothelia Growth Medium-2 (EBM-2)

Endothelia growth medium-2 was supplemented with 10% (v/v) heat-inactivated FCS (30 minutes, 65°C Additionalyl, it BulletKit™ was added for cultivation of HUVECs from PromoCell then called EGM-2.

2.5.2. Brain-Heart-infusion (BHI) medium

2.5.2.1. BHI-bouillon

BHI	37 g
ddH_2O	ad 1000 ml

The medium was autoclaved (15 minutes, $2*10^5$ Pa at 121°C) before use.

2.5.2.2. BHI-agar

BHI included agar	52 g
ddH_2O	1000 ml

The medium was autoclaved (15 minutes, $2*10^5$ Pa at 121°C) before use.

2.6. General buffers and solutions

2.6.1. 1% agarose gel

Agarose	1.0 g
1x TAE	100 ml

2.6.2. 50x TAE buffer

Tris-HCl	242.0 g
EDTA	18.60 g
Glacial acetic acid	57 ml
dH_2O	ad 1000 ml

The buffer was autoclaved (15 minutes, $2*10^5$ Pa at 121°C).

2.6.3 Cacodylate buffer

Cacodylate	13.8 g
$CaCl_2$	1.29 g
$MgCl_2$	0.95 g

The pH was adjusted to pH 6.9.

2.6.4 PBS

Sodium chloride	8.0 g
Disodium hydrogen phosphate	1.4 g
Potassium dihydrogen phosphate	1.0 g
Potassium chloride	1.0 g
ddH_2O	ad 1000 ml

The pH was adjusted to 7 with NaOH and the buffer was then autoclaved (15 minutes, $2*10^5$ Pa at 121°C).

2.6.5. Solution C

BSA	0.50 g
NaCl	0.88 g

The pH was adjusted to pH 7.4.

2.6.6. Solution for cell lysis (RLT buffer)

10 µL β-mercaptoethanol were added to 1 ml RLT buffer
(Ready to use from RNeasy® Mini Kit QIAGEN)

2.6.7. Solutions for ELISA

2.6.7.1. Coating buffer for IL-1β, IL-6 and TNF-α

Sodium carbonate	10.6 g
ddH_2O	ad 1000 ml

The pH was adjusted to 9.5.

2.6.7.2. Coating buffer for KC and MIP2

1x PBS

2.6.7.3. Coating buffer for IL-12 p70 and IL-10

Di-sodium hydrogen-phosphate	14,1960 g
ddH_2O	ad 1000 ml

The pH was adjusted to 7.5.

2.6.7.4. Blocking buffer

1x PBS with 10% FCS

2.6.7.5. Washing buffer

1x PBS with 0.05% Tween20

2.6.8. Solutions for β-hexosaminidase assay

2.6.8.1 Citrate buffer

Citric acid	7,6850 g

Na2HPO4·7H2O	5,3620 g
Glycine	30,027 g
Triton X-100	0,1 ml

2.6.8.2 PNAG

PNAG	5g/l

PNAG was dissolved in citrate buffer. Due to poor solubility of PNAG in citrate buffer, shacking and mild heating (37-40°C) of PNAG were required for complete dissolving of PNAG This process can take up to 3h. The solution is stable for 24h.

2.6.8.3. Tyrode's buffer

1M HEPES	10 ml
Sodium chloride	7.540 g
Potassium chloride	0.370 g
Calcium chloride	0.206 g
Magnesium chloride	0.203 g
Glucose	1.008 g
BSA	1.000 g
Heparin	100 µg/ml
ddH_2O	ad 1000 ml

The solution was filtered through a 0.2 µm filter and stored at 4°C.

2.6.9. TE buffer

Tris-HCl (pH 8.0)	20 mM
EDTA	1 mM
ddH_2O	100 ml

The solution was autoclave (15 minutes, $2*10^5$ Pa at 121°C) before use.

2.7. Growth factors and antibiotics

GM-CSF	BIOLEGEND
*m*SCF	CELL SIGNALING
*m*IL-3	BIOLEGEND
*m*IL-4	BIOLEGEND
Doxycycline	SIGMA

Erythromycin	INVITROGEN
Gentamicin	GIBCO
Penicillin	INVITROGEN
Streptomycin	INVITROGEN

2.8. Kits

DNA-free™ Kit for DNA depletion	AMBION
High Capacity cDNA Reverse Transcription kit	APPLIED BIOSYSTEMS
LIVE/DEAD^{r} BacLight^{TM} Bacterial/Viability Kit	INVITROGEN
RiboPure Bacteria RNA Extraction Kit	AMBION
RNeasy ® Mini Kit	QIAGEN
SensiFAST™ SYBR & Fluorescein One-Step Kit	BIOLINE

2.9. Bacteria strains

Table 6. *E .faecalis* strains and other bacteria

ATCC strain No. / internal	Source	Reference
47077 / *E. faecalis* OG1RF	ATCC	
47077+pMV158*gfp* / *E. faecalis* GFP	Kindly provided by NIETO, C. and ESPINOSA, M.; Madrid, Spain	[154]
***Staphylococcus aureus* SH1000**	Kindly provided by FOSTER, S.; Sheffield, England	[155]
98579 / *Escherichia coli* TOP10	ATCC	

2.10. Bacterial culture

2.10.1. Growth conditions

E. faecalis stock cultures were maintained at –80°C, and cultured at 37 °C in BHI-medium for 6 h. *S. aureus* stock cultures were maintained at –80°C, and cultured at 37 °C in BHI-medium for 6 h. *E. coli* stock culture were maintained at –80°C, and cultured at 37 °C in LB-medium for 6 h.

2.10.2. Inoculum preparation

Bacteria were collected at mid-log phase (absorbance at OD_{600nm}~0.5) and harvested by centrifugation for 10 minutes at 4,000 rpm (HERAEUS MEGAFUGE 1.0). Bacteria were washed once with sterile 1x PBS and adjusted to 15% transmission at 600 nm (PHARMACIA LKB Novaspec II) using sterile 1x PBS. This is equal to 10^9 bacteria/ml. To heat-kill bacteria, bacteria were boiled for 1h at 95°C. Bacteria suspensions were further diluted in cell culture medium depending on cell type or 1x PBS for *in vivo* infection experiments to achieve the required inoculum concentration. For *in vitro* infection experiments, a multiplicity of infection (MOI) of ten bacteria per eukaryotic cell (10:1) was used, except were indicated differently.

2.11. Preparation and culture conditions of eukaryotic cells

2.11.1. Preparation and culture of primary *murine* bone-marrow derived mast cells (BMMCs)

Mouse bone-marrow-derived mast cells were used from bone marrow extruded from the femur and tibia of C57BL/6 wild-type, TLR2 knock-out mice or MyD88 knock-out mice. Bone marrow was flushed out of the bones by using a 23G syringe with warm complete IMDM and cultured in the presence of *murine* IL-3 (*m*IL-3,BIO LEGEND) at a concentration of 5 ng/ml, supplemented with 100µg/ml penicillin/streptomycin for 3 weeks at 37 °C, 5% CO_2 (FORMA scientific water mantled incubator 3111). After 3 weeks, the differentiation status of BMMCs was checked by FACS analysis using the surface marker CD117. Cells were used if at least 95% purity regarding CD117 expression was achieved. Mast cell culture was maintained with *murine* steam cell factor (*m*SCF, CELL SIGNALING) in a concentration of 50 ng/ml instead of murine IL-3 for up to 6-8 weeks

Cells were harvested, counted (Neubauer chamber), adjusted to 1x 10^6 per ml and used for experiments, except where noted differently.

2.11.2. Preparation and culture of primary *murine* bone marrow derived dendritic cells (BMDCs)

Murine bone marrow dendritic cells were used from bone marrow extruded from the femur and tibia of Balb/c wild-type mice or TLR2 knock-out or MyD88 knock-out mice with a C57BL/6 background. Bone marrow was flushed out of the bones by using a 23G syringe with warm complete RPMI and cultured in the presence of *murine* IL-4 (*m*IL-4, BIOLEGEND) at a concentration of 10 ng/ml and 50 ng/ml granulocyte-macrophage colony-

stimulating factor (GM-CSF, BIOLEGEND) supplemented, with 100 µg/ml penicillin/streptomycin for 8 days at 37°C, 5% CO_2 (FORMA scientific water mantled incubator 3111). After 8 days the cells were enriched using an OptiPrep^{TM} gradient according to the manufacturer's instructions.

The differentiation status of BMDCs was checked by flow cytometry analysis using the surface marker CD11c^{high}. Cells were used if at least 90% purity regarding CD11c expression was achieved.

Cells were harvested, counted (Neubauer chamber), adjusted to 1x 10^6 per ml and used for experiments, except where stated differently.

2.11.3. Culturing of primary human umbilical vein endothelia cells (HUVECs)

HUVECs were sub-cultured when 80% confluence was reached. For sub-cultivation, cells were washed twice with 4 ml warm HEPES-BSS (SIGMA) to remove dead cells, cell debris and medium components. Then 3 ml Trypsin/EDTA (SIGMA) were added to detach cells from the cell culture dish. Trypsin treatment was stopped by the addition of 3 ml Trypsin-Neutralization Solution (TNS) (SIGMA). The detached cells were carefully removed from the petri dish, centrifuged 10 min at 180 *x* g and resuspended in 4 ml fresh EGM-1 for HUVEC from LONZA, EGM-2 for HUVEC from PROMOCELL or ECM for HUVEC from SCIENCELL. Harvested cells were counted (Neubauer chamber), adjusted to 5 x 10^4 per ml in 10 cm culture-dishes (SARSTEDT) and incubated at 37°C and 5% CO_2 until passage five or used for experiments, except described differences. The media was changed every 3-4 days.

2.12. Experimental procedure

2.12.1. Infection of murine primary cells

Bone marrow-derived cells were taken and centrifuged for 5 min at 180 *x* g. The supernatant was discarded and the pellet was resuspended in 10 ml of fresh medium. The cells were counted in a "Neubauer" chamber and the concentration of cells was determined. The cells were adjusted to a final concentration of 1x10^6 cells/ml in DMEM medium. Then the cells were seeded into a 4-well culture plate and incubated for 2h at 37°C and 5% CO_2. After 2h the cells were infected with *E. faecalis* at a multiplicity of infection (MOI) of 10:1 for immunohistochemistry assays or microscopy. For killing assays a MOI of 1:1 was used. For electron microscopy a MOI of 25:1 was used.

2.12.2. Infection of HUVECs

HUVECs were prepared as described in 2.11.3. The cells were counted in a "Neubauer" chamber and the concentrations of cells were determined. Cells were adjusted to a final concentration of $5x10^4$ cells/well in 500 µl culture medium and seeded into a 4-, 12- or 24-well plate until a confluent monolayer was observed. Cells were washed twice with 1x PBS and fresh EBM-2 containing 5% FCS was added. The cells were infected with *E. faecalis* at a MOI of 10:1. For electron microscopy a MOI of 25:1 was used.

2.12.3. Killing assay of intracellular enterococci by dendritic cells

Dendritic cells were infected as described in 2.12.1 and incubated at 37°C, 5% CO_2 and 95% humidity. After 2h antibiotics were added to samples. After 2h, 4h and 24h samples were taken, centrifuged at 180 *x* g for 10 min and lysed with 300 µL Triton-X100 for 5 min. 30 µL were taken and 20 µL were plated in a dilution series of 1:1, 1:2, 1:4, 1:8 and 1:16 of BEA-agar plates. The plates were incubated at 37°C for 18 h and the CFU was counted.

2.12.4. Killing assay of extracellular enterococci by mast cells

Mast cells were infected as described in 2.12.1 and incubated at 37°C, 5% CO2 and 95% humidity. After 30, 60 and 180 min samples of 30 µL were taken and 20 µL were plated in a dilution series of 1:1, 1:2, 1:4, 1:8 and 1:16 of BEA-agar plates. The plates were incubated at 37°C for 18 h and the CFU was determined.

2.12.5. Mast cell extracellular traps killing

To induce mast cells extracellular trap formation (MCET) mast cells were pre-treated with 10 µg/ml PMA for 3 h. Subsequently, the cells were treated with 5 µg/ml cytochalasin D for 30 minutes to inhibit cellular phagocytosis of cells that have not induced MCETs.

Mast cells were infected as described in 2.12.1 with a MOI of 1:1 and incubated at 37°C, 5% CO_2 and 95% humidity. After 30, 60 and 180 min samples of 50 µL were taken and plated in a dilution series of 1:1, 1:2, 1:4, 1:8 and 1:16 of BEA-agar plates.

2.13. Microscopy

2.13.1. Electron microscopy.

For scanning electron microscopy, samples were fixed with 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer for 1 h on ice and washed in TE. Dehydration was performed with a graded series of acetone, critical-point dried with CO2, and sputter coated with gold before examination in a Zeiss field emission scanning electron microscope (DSM982 Gemini) at 5 kV using the Everhart-Thornley SE detector and the in-lens secondary electron (SE) detector in a 50:50 ratio.

For transmission electron microscopy infected cells were washed twice with 0.1 M cacodylate buffer. Cells were fixed with 0.1 M cacodylate buffer + 5% paraformaldehyde + 2% glutaraldehyde and simultaneously contrasted with 1% aqueous osmium tetroxide for 1h at room temperature (RT). Afterwards, samples were washed with 0.1 M cacodylate buffer and dehydrated in a grade series of ethanol (10%, 30%, 50%, 70% and 90%) with 30 min on ice for each step. Following incubation with 100% ethanol for 30 min at RT, samples were embedded in Spurr resin (Spurr, 1969) and polymerized at 70°C f or 16 h. After polymerization ultra-thin section of infected cell layers were cut with a diamond knife. Sections were collected onto butvar-coated Cu-grids, contrasted with 4% uranyl-acetate (UAc) for 3 min at RT and lead-citrate for 15 sec at RT. Following a final washing step with ddH_2O, sections were air dried and examined with a TEM EM 910. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024 x 1024) with ITEM-Software (Olympus Soft Imaging Solutions). Images were taken by Prof. Dr. M. Rohde (HZI Braunschweig).

2.13.2. Double-immunofluorescence (DIF) microscopy

500 µL eukaryotic cell suspensions (BMDC& BMMC: equal to $5x10^5$ cells/well; HUVEC: equal to $5x10^4$ cells/well) were added in a 4 or 24 culture well plate containing cover-slides. *Murine* cells were infected as described in 2.12.1 and HUVECs were infected as described in 2.12.2. Cells were incubated at 37°C, 5% CO_2 and 95% humidity. The cells were washed twice with 1x PBS and fixed with 3% PFA for 15 min at RT. Then cells were washed twice with 1x PBS and blocked with 200 µL/well 1x PBS 10% FCS (h.i.) for 30 minutes. The blocking solution was discarded and 20 µL/well of a 20 µg/ml solution diluted in 1x PBS 10% FCS (h.i.) of rabbit anti-*E. faecalis* antibody was added for further incubation for 30 minutes at RT. After incubation the cells were washed twice with 1x PBS and the second antibody was added (20 µL/well of a 20 µg/ml solution diluted in 1x PBS 10% FCS (h.i.) of goat anti-rabbit IgG *Alexa-Flour 488*) and incubated for 30 min at RT. Then the cells were washed twice with 1x PBS and permeabilized using 200 µL 0.1% Triton-X 100 in 1x PBS per

well for 5 min at RT. In case of HUVECs an actin-staning with *Alexa-Fluor 488* Phalloidin, 20 µL/well of a 20 µg/ml solution diluted in 1x PBS 10% FCS (h.i.) for 30 min was performed. After two washing steps with 1x PBS, the third antibody was added (20 µL/well a 20 µg/ml solution diluted in 1x PBS 10% FCS (h.i.) of rabbit anti-*E. faecalis* antibody) and incubated for 30 min at RT. The cells were washed twice again with 1x PBS and incubated with the last antibody (20 µL of a 5µg/ml solution of a goat anti-rabbit IgG *Alexa-Flour 568* antibody diluted in 1x PBS 10% FCS (h.i.)) for 30min at RT. The cells were washed twice with 1x PBS and the cover-slides were placed on a slide with 5 µL Moviol. After 1 h the cover-slides were sealed with nail polishing and used for microscopy.

2.13.3. FITC-labeling of *E. faecalis*

E. faecalis inoculum was cultured as described under 2.10.1. Carboxyflourescein was added to bacteria suspension in a concentration of 0.2 mg/ml and incubated for 30 min at 4°C in the dark. Then, the cultures were centrifuged at 1,900 *x* g for 10 min and washed in 1x PBS until the supernatant was colorless.

2.13.4. LIVE/DEAD stain

250 µL mast cells ($2{,}5x10^5$ cells/well) were added in a culture well plate, which contained a poly-l-lysine treated cover-slide. Mast cells were infected as described under 2.12.1 and incubated at 37°C, 5% CO_2 and 95% humidity. The cells were washed twice with 1x PBS.
500 µL fresh DMEM medium was added. 5 µL each of the two compounds of LIVE/DEAD^R BacLight^{TM} Bacterial/Viability Kit (INVITROGEN) were mixed 1:2. 2 µL of the mixed staining solution was added to each sample and incubated for 15 min at RT. After incubation the samples were washed in 1x PBS and the cover-slides were placed on a slide with 5 µL Moviol. After 2h the cover-slides were sealed with nail polishing and used for microscopy

2.13.5. Toluidine stain

250 µL of mast cells ($5x10^5$ cells/well) were infected as described in 2.12.1 and incubated at 37°C, 5% CO_2 and 95% humidity. After 2h the mast cells were fixed with 3% PFA for 10 min at RT. 100 µL of cell suspension was centrifuged on slides at 150 *x* g for 5 min and dried on air at RT. 5 µL of Toluidine-blue (0.5% in 1x PBS) was spotted on the slides and incubated for 5 min at RT. Subsequently, the slides were washed with water until the blue color disappeared.

2.14. Immunohistochemistry

2.14.1. Measuring Mast Cell Mediator Release (β-Hexosaminidase)

Mast cells were resuspended in the TYRODES buffer and aliquoted into 96-well plates (BMMCs 90.000/well) in a final volume of 100 µL. Cells were incubated in 37 °C for 5–10 min to equilibrate the cells to the activation temperature.

10× stocks of agonists or inhibitors (antagonists) were prepared in TYRODES buffer. 10 µL of these stocks were added to each well. The plate was incubated in a heating block or warm air oven at 37 °C for 30 min (without CO_2). 100 µL of PNAG solution was transferred into two new 96-well plates using a multichannel pipette. One plate is for measurement of secreted β-hexosaminidase activity (supernatants) and the other is used to calculate the total amount of supernatants and lysates to determine the percentage release. After incubation, the plate was centrifuged at 450 *x* g, at 4 °C for 5 min to stop the reaction and to ensure the cells are sediment to the bottom of the wells. The plate was tilt to an angle of 45° and carefully 50 µL aliquots of the cell-free supernatant was removed from the top using a multichannel pipette where the depth of the solution is greatest. The aliquots were added into the PNAG solution and the plate was incubated for 90 min at 37 °C (without CO2). It is important that the cells are not transferred into PNAG solution. In order to calculate the total β-hexosaminidase activities, add 150 µL of 0.1% Triton X-100 solution into the 50 µL of supernatants and cells remaining in the original incubation plate. Resuspend carefully and take 50 µL of lysates and add into the PNAG solution and incubate the plate for 90 min at 37 °C (without CO2). After incubation, add 50 µL 0.4 M Glycine buffer into each well. The appearance of yellow color indicates the extent of β-hexosaminidase activity. Read plate absorbance at 405 nm with reference filter at 620 nm. Calculate the percentage of hexosaminidase activities present in the supernatants. The protocol is adapted and modified from [156].

2.14.2. ELISA

The concentration of different cytokines and chemokines was determined by using commercial ELISA systems (BD Pharmingen). For this purpose, a 96-well plate was coated

with the respective anti-mouse antibody for TNF-α; IFN-γ & IL-6 diluted 1:250 in 0.1M Na_2HCO_3 or MCP-1 & IL-12p70 1:250 in 0.2M Na_2PO_4 or KC & MIP2 1:180 in 1x PBS at 4°C overnight which is different to the manufactures manual. To detect cytokines and chemokines from human cells, anti-human antibody for IL-1β and IL-8 were diluted 1:250 in 0.1M Na_2HCO_3 at 4°C overnight which is different to the manufactures manual. The wells were washed three times with 0.05% Tween in 1x PBS (PBST) and blocked with 10% FCS-PBS for 1h. Then, the wells were washed again with PBST and samples and standards were added for 2h. After incubation for 2h, the wells were washed again and a biotinylated anti-mouse or anti-human antibody was added as detection antibody 1:250 in 10% FCS-PBS followed by incubation for 1h. After washing with PBST streptavidin-peroxidase 1:250 in 10% FCS-PBS was added for 30 min at RT. After the final PBST washing step, the ELISA was developed using 50 µL TMB per well. To stop the reaction 25 µL of 2N sulfuric acid was added. A standard curve was plotted for each cytokine using the corresponding recombinant protein as standard. The concentration was calculated based on the generated standard curve measuring the absorption at 450 nm against a reference wavelength at 570 nm (TECAN Sunrise).

2.14.3. Flow cytometry

To determine the purity of a cell culture, flow cytometry analysis was used. Briefly, 200 µL of the cell culture sample was taken from a culture, and the FcγIII/II receptors were blocked with 0.25 µg/ml mouse anti-FcγIII/II antibody for 5 min at RT. To stain the CD117 c-kit receptor on the mast cell surface or the CD11c receptor on the surface of DCs, the cell suspension was incubated with 2µg/ml of anti-CD117 c-kit antibody or anti-CD11c antibody for 1 h at 4°C. After incubation, the cells were washed with 2.5 ml 10% FCS (h.i.) in 1x PBS and centrifuged at 180 *x* g for 5 min. Dendritic cells infected as described in 2.12.1 were also measured for CD40; CD80 and CD86. Dendritic cells were double-stained with anti-mouse CD11c-PE antibody and one additional surface marker (2µg/ml in DMEM for 1h at 4°C). HUVECs infected as described in 2.12.2 were measured for CD54, CD62-E and CD106. HUVECs were only single stained with 2 µg/ml of anti-CD54, anti-CD62-E and anti-CD106 antibody for 1h in EBM-2 at 4°C. The supernatant was discarded and the sample was measured with a BD Calibur or BD LSR II (BD BIOSCIENCE). An unstained sample was used as negative cell population.

2.15. Molecular biology methods

2.15.1. Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis in an electric field. Unless otherwise specified, the separation was carried out in a 1% agarose gel (in TAE buffer) at 100 V and 500 mA for 45 min. Afterwards, the gel was stained in a 0.001% ethidium bromide solution for 10 min. Gene Ruler DNA Ladder Mix (FERMENTAS) was used as a size standard.

2.15.2. cDNA synthesis

cDNA was synthesized by using the High Capacity cDNA Reverse-Transcription-Kit (APPLIED BIOSYSTEMS). Each step was performed on ice or at 4°C. Briefly, 1 µL RNA (1-3µg) were incubated with 2 µL Oligo(dT)15 and 10 µL DEPC-water at 70°C for 10 min and further for 2 min on ice. 8 µL master mix was added (4 µL 5xFirst strand-Puffer; 2 µL 100mM DTT; 1 µL 10mM dNTPs; 0.4 µL Rnasin; 0.6 µL DEPC-water) and incubated for 2 min at 42°C. After 2 min 1 µL *reverse* transcriptase was added and further incubated for 30 min. 10 µL TE buffer were added and the samples were incubated for 2 min at 90°C and then stored on ice. For long-term storage samples were kept at -80°C.

2.15.3. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

2.15.3.1. Preparation

Murine primary cells were infected as described in 2.12.1. Human cells were infected as described in 2.12.2. Samples were taken after 6 h and 24h, non-infected cells served as controls. For qRT-PCR, β-actin was used as housekeeping gene and internal control for normalization of the cDNA amount. cDNA was analyzed for differential gene expression. The used primers are listed in Table 3 for mouse genes and Table 4 for human genes. The reaction mixture was prepared with the SensiFAST™ SYBR & Fluorescein One-Step Kit (BIOLINE) as shown in table 8.

Table 7. qRT-PCR reaction mixture

qRT-PCR--Master mix	µl	Final concentration	Reduced approach [µl]
2x SensiFAST Mix	10	1x	6.25

qRT-PCR--Master mix	µl	Final concentration	Reduced approach [µl]
10µM F-Primer	0.8	400nM	0.5
10µM R-Primer	0.8	400nM	0.5
RT	0.2		0.125
RiboSafe RNase Inhibitor	0.4		0.25
Water (DEPC)	3.8		2.375
Template	4		2.5
Total volume	**20**		**10**

The RT reaction was performed on a Rotor-Gene Q (QIAGEN).

Table 8. Cycling program

2-Step Cycling program			
1x	45°C	15min.	for RT
1x	95°C	5min.	activation of polymerase
40x	95°C	20sec	denaturation
	... °C	20sec	annealing
	72°C	20sec	extension
1x	55 – 92 °C	∞	melting

2.15.3.2. Analyze of qRT-PCR

The data was calculated using $2^{-\Delta\Delta CT}$ of the Pfaffl equation [157] and expressed as a ratio of the relative mRNA expression in infected samples compared to the uninfected controls.

2.15.4. RNA Extraction

For infections, $1x10^6$ murine cells in 1 ml DMEM medium were infected as described in 2.12.1. For human cells, $1x10^5$ cells in 1 ml EBM-2 medium were infected as described in 2.12.2.. After 6 h and 24h, the cells were collected by centrifugation (180 *x* g, 10min, RT) and the supernatant was discarded. The cell pellet was washed in 1x PBS and centrifuged again. The supernatant was discarded and the pellet resuspended in 350 µL RLT buffer supplemented with 10 µL β-mercaptoethanol. The RNA extraction was performed by using the RNeasy Mini Kit (QIAGEN) according to the manufacturer´s instructions. Total RNA was isolated so prokaryotic and eukaryotic RNA could be detected. Afterwards a DNase treatment

(AMBION) was done according to the manufacturer´s instructions to completely remove DNA.

2.16. Experiments with mice

2.16.1. Animal husbandry of mice

8 weeks-old female C57BL/6 mice or Balb/c were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were housed in microisolator cages and were given food and water *ad libitum*. All experiments were performed in accordance with protocols approved by the local animal ethical committee board.

2.16.2. Euthanasia of mice

The mice were killed painlessly by gassing with carbon dioxide.

2.16.3. Intraperitoneal (IP) infection in mice

For intraperitoneal infection a mouse was placed on the cage lid and fixed in the neck. The animals were maintained with the head down during the infection, in order to relieve the abdominal cavity. The cannula (0.45 x 12mmB, Braun) was introduced approximately 1 cm in the lateral abdominal and 500 µL of a $5x10^8$ bacterial suspension was injected into the peritoneal cavity.

2.16.4. Peritoneal lavage

At different time points post infection the mice were killed as described in 2.16.2. The abdominal cavity was washed twice with exactly 5 ml of DMEM medium, removed and transferred to a Falcon tube. Cells collected during lavage were centrifuged at 180 *x* g and 4°C for 5 min. The cells were counted and stained for flow-cytometry as described in 2.14.3. The samples were analyzed with FLOWJO v7.6.5.

2.17. Statistical analysis

All experiments were done at least three times unless otherwise stated. A comparison between groups was performed by using GraphPad Prism 5.0 with one-way Anova non-parametric

Friedman test or Wilcoxon test. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1 Characterization of virulence-associated genes in *Enterococcus faecalis* strains from different clinical disease manifestations

The reasons for the change in epidemiology and clinical manifestation of enterococcal-associated diseases as well as the increasing role of these microorganisms as a source of nosocomial infections still remain unknown. Whether this rise in the incidence of severe enterococcal infections resulted from changes in virulence properties of the bacteria and/or increased host susceptibility to factors produced by reemerging enterococcal strains due to the lack of protective immunity remains an active area of research. These possibilities are not mutually exclusive, and there is little doubt that the disease outcome is determined by host-pathogen interplay.

E. faecalis is able to cause a variety of different diseases ranging from urinary tract, wound to soft tissue infections as well as sepsis and endocarditis [19-21]. To investigate if the presence of certain virulence factors is correlating with disease manifestation, the presence of certain virulence genes in various isolates representing different disease outcomes have been investigated. Five strains were collected from patients that developed endocarditis: six strains from patients with infections resulting from artificial hip joint transplantation and eight strains from patients with serious blood infections as well as one case of bone infection caused by *E. faecalis* have been used for this study.

No specific correlation between the presence of virulence genes and disease manifestation could be determined (see table 10, appendix). The abundance of virulence genes in all investigated strains showed only moderate differences among the different strains. These findings argue for either the influence of the regulation of the expression of virulence factors on one side but also for an influence of host immune factors, which are involved in disease manifestation, progression and resolution of infections caused by this opportunistic pathogen on the other side. This finding strengthens the importance to investigate the influence of host immune mechanisms in the pathogenesis of *E. faecalis* induced infections.

3.2. Response of mast cells (MCs) during infection with *E. faecalis*

One important aim of this study was first to characterize the role of MCs, an innate immune cell population abundantly present beneath the intestinal mucosal barrier, in response to *E. faecalis* and second the specific aspects of the signaling pathways involved in this response. The role of TLR2-MyD88 signaling pathway, one of the most important signaling pathways

for immune recognition of Gram-positive was explored for MCs to *E faecalis* infection. A central finding was that MCs can contribute significantly by extracellular killing mechanisms to the control of *E. faecalis*. In addition, the response of MCs to *E. faecalis* includes degranulation and release of inflammatory cytokines dependent on TLR2-MyD88 signaling.

3.2.1. *E. faecalis* shows a strong association to the surface of MCs *in vitro* and is able to induce MC degranulation

One of the extracellular interactions between MCs and *E. faecalis* consist in the extracellular bacterial association of the bacteria to the surface of MCs (FIG 3.1. A). Furthermore, electron microscopic investigations reveal a strong induction of MC degranulation in response to *E. faecalis* characterized by a rapid release of pre-stored granules into the extracellular environment (FIG 3.1. B).

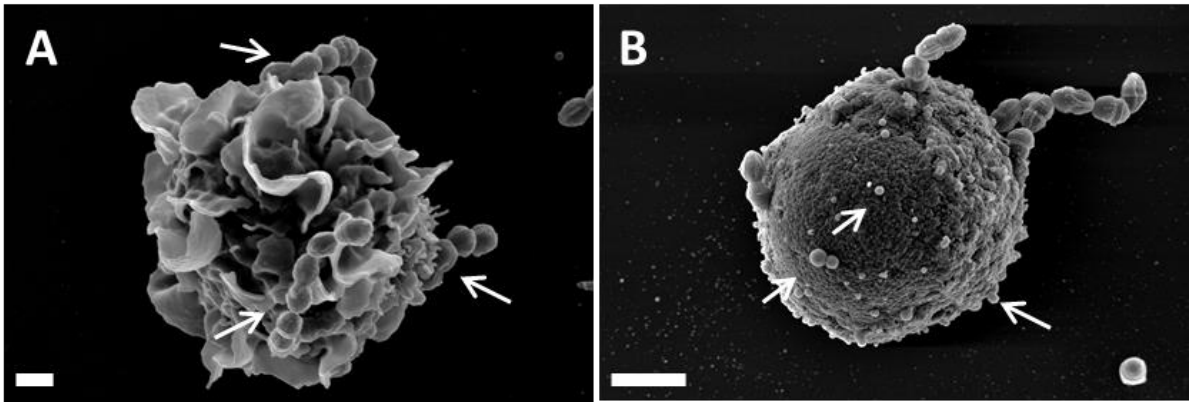

FIG 3.1.: Interaction of *E. faecalis* with MCs. (A) Association of *E. faecalis* on the surface of MCs 2h post infection indicated by white arrows. (B) MCs degranulation in response to *E. faecalis*. Release of granules is indicated by white arrows. Bar represents (A) 1µm, (B) 2µm. SEM images by Prof. Dr. Manfred Rohde, Helmholtz Center for Infection Research.

3.2.2. MCs are able to limit the bacterial growth of *E. faecalis*

As MCs have been previously shown to inhibit the growth of several important human pathogens [90, 97], it was determined if MCs were also capable to modulate the growth of *E. faecalis in vitro*. For this purpose, the growth of *E. faecalis* was determined over time in the presence and absence of MCs. The results depicted in Fig. 3.2 A. show that MCs were also very efficient to suppress the growth of *E. faecalis*. MCs were capable to inhibit the growth

over a wide range of clinical isolates and no correlation between growth inhibition and clinical presentation could be found (Fig. 3.2. B).

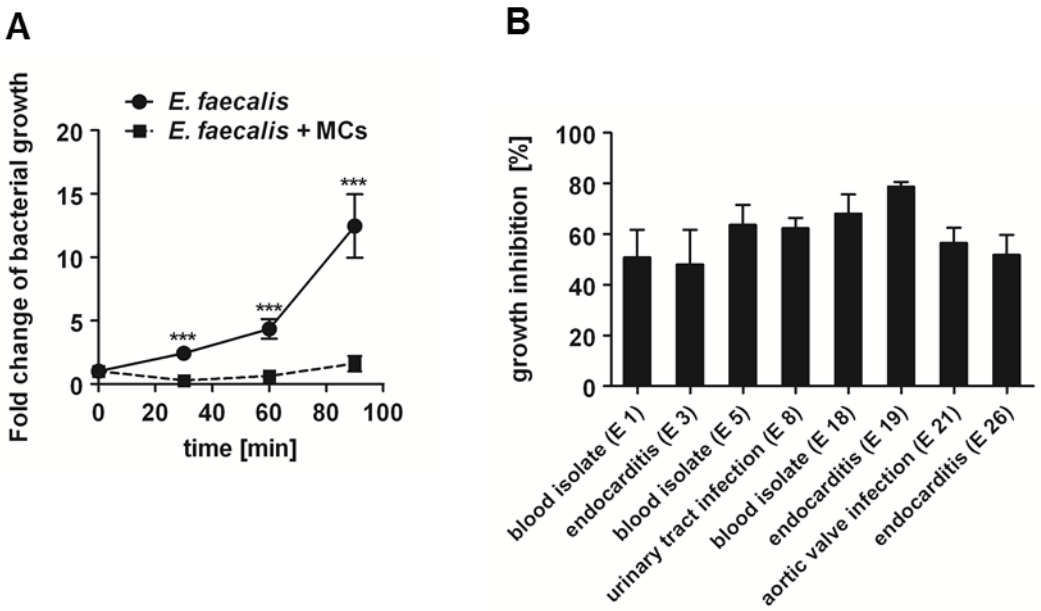

FIG 3.2.: Growth inhibition of *E. faecalis* by MCs. (A) MCs were infected as described in section 2.12.1 with *E. faecalis* strain OG1RF over a 90 min time course. After 30, 60 and 90 min the CFU was determinate by plating serial dilutions on bile-esculin agar plates and compared against a growth control of bacteria without MCs. (B) Growth inhibition of different clinical isolates in percent using the following formula: [(CFU in medium alone - CFU in medium with MCs) / CFU in medium alone]*100. Each bar represents the mean SD of triplicates from three independent experiments. ***, $P < 0.001$.

3.2.3. MCs contribute to the growth inhibition of *E. faecalis* by various extracellular mechanisms

It has previously been shown that growth inhibition elicited by MCs in response to bacterial pathogens is mediated by the release of MCETs that are antimicrobial [97]. The release of MCETs upon encounter of *E. faecalis* was determined. For this purpose, MCs were infected for 3h with *E. faecalis*, fixed, and processed for immunofluorescence microscopy using bacterial Live/Dead® staining. Live bacteria appear in green and dead bacteria in red. The MCETs were counterstained with the DNA dye DAPI as DNA is the backbone of MCETs. It could be clearly shown that MCs release MCETs in response to *E. faecalis* (FIG. 3.3. A). Interestingly, many bacteria that were trapped within MCET structures appeared green and seem to be alive (FIG 3.3. A and B). In addition, bacteria that were not in contact with these

structures appear red. This finding gives evidence that a second mechanism is responsible for direct killing of *E. faecalis* by MCs.

To determine the proportional contribution of MCETs to the growth inhibition of *E. faecalis*, MCs were infected with *E. faecalis* at a MOI of 1:1 for 60 min in the presence or absence of 50 mU endonuclease to degrade the DNA backbone of MCETs [97]. This digestion of MCETs resulted in only partial growth inhibition of *E. faecalis* by MCs suggesting that also degranulation contributes to killing of *E. faecalis* (FIG 3.3. C). To confirm this postulation, the levels of growth inhibition of *E. faecalis* by MCs was determined in degranulated (cromolyn-treated) MCs and MCs, in which MCETs have been dismantled by nuclease treatment. MCs treated with both - cromolyn and nuclease - were completely impaired in their capacity to inhibit the growth of *E. faecalis* (FIG 3.3. C). These results confirmed the contribution of both the release of MCETs and release of granules to the antimicrobial effect of MCs against *E. faecalis*.

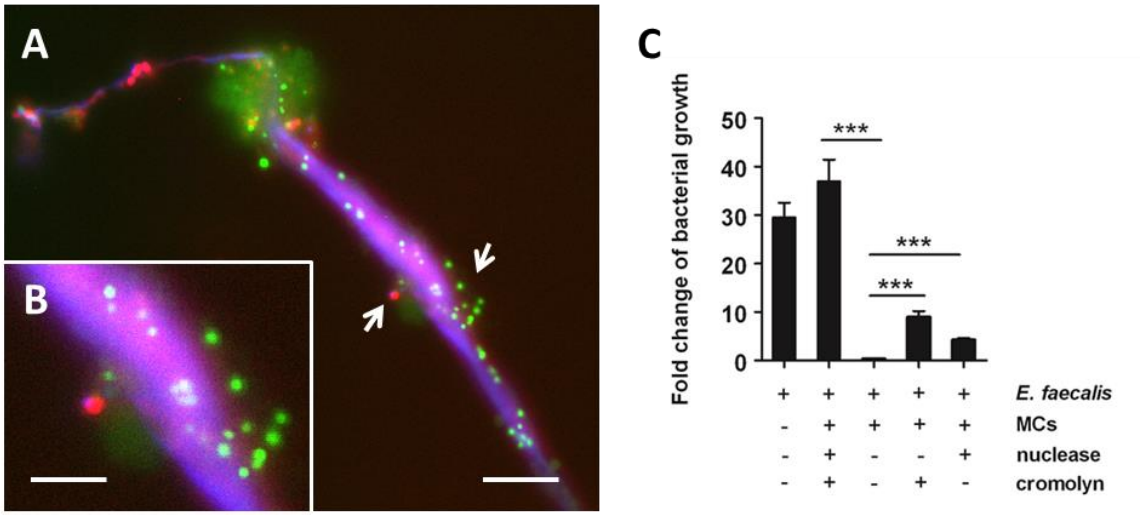

FIG. 3.3.: Live/Dead® staining of MCs infected with *E. faecalis* at an MOI of 10:1. (A and B) Immunofluorescence microscopic overview of mast cells releasing MCET in response to *E. faecalis.* Dead bacteria (red color) as well as live bacteria (green color), could be found in association with MCETs (blue color). (B) Magnification of area in (A) indicated by arrows. (C) Growth of *E. faecalis* in medium alone or co-cultured with MCs either untreated or treated with cromolyn (to block degranulation), nuclease (to dismantle MCET structures) or both for 90 min. Each point represents the mean ± SD of triplicates from three independent experiments. ***, $p < 0.0001$

3.2.4. Role of TLR2-MyD88 signaling pathway in immune response of MCs to *E. faecalis*

3.2.4.1. Release of MCETs is not depending on the TLR2-MyD88-signaling pathway

Immunofluorescence microscopy was performed to examine if and to which extent TLR signaling is involved in the formation of MCETs after encounter of *E. faecalis*. MCs derived from wild type, TLR2 $^{-/-}$ and MyD88 $^{-/-}$ mice were infected with *E. faecalis* at an MOI of 1:1 for 3h and extracellular traps were stained by DAPI. The number of MCETs per cell has then been counted microscopically in ten independent fields of view. No differences in the formation of MCETs were observed among the different knock out cells compared to the wild-type cells demonstrating that TLR/MyD88 signaling is not involved in this particular extracellular response of MCs towards *E. faecalis*.

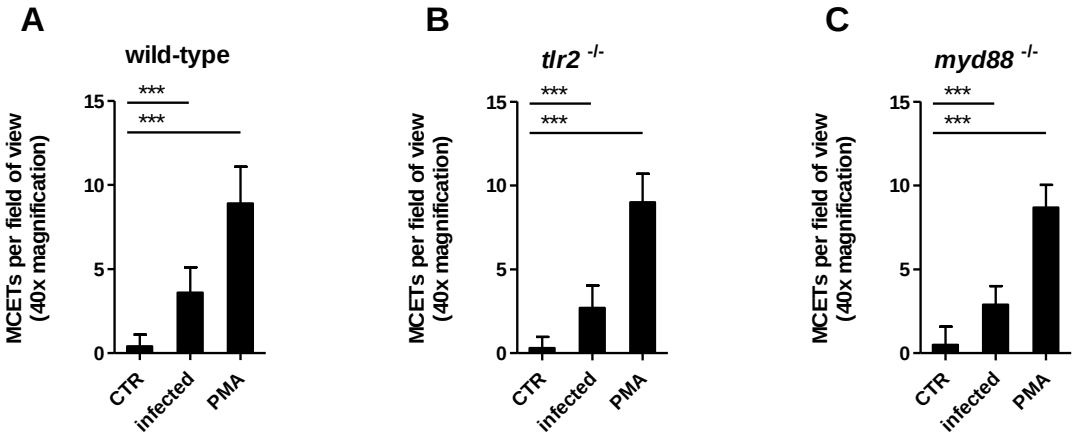

FIG 3.4.: Release of MCET is TLR-MyD88-pathway independent. Numbers of MCETs released during *in vitro* infection with *E. faecalis* were counted in ten independent fields of view. No significant difference between wild-type, TLR2 $^{-/-}$ or MyD88 $^{-/-}$ MCs could be obtained. Each bar represents the mean ± SD of microscopical counts of ten independent fields of views. ***, $P < 0.001$.

3.2.4.2. TLR2 signaling is crucial for cytokine response of MCs after recognition of *E. faecalis*

To investigate the cytokine response of MCs to *E. faecalis*, the release of IL-6 and TNF-α of MCs derived from wild-type or mice deficient in TLR2 or MyD88 was measured by ELISA.

In an *in vitro* infection it could be demonstrated that the amount of IL-6 and TNF-α is significantly lower in MCs derived from MyD88 knockout mice than those derived from MCs of wild-type origin (FIG.3.5.).

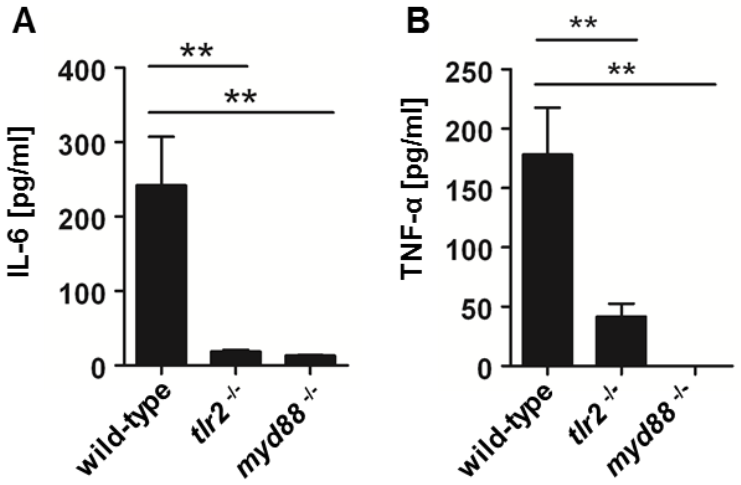

FIG 3.5.: Cytokine profile of MCs derived from wild-type TLR2 $^{-/-}$ or MyD88 $^{-/-}$ mice in response to *E. faecalis*. MCs derived from wild-type TLR2 $^{-/-}$ or MyD88 $^{-/-}$ mice response with a lower release of IL-6 (A) and TNF-α compared to cells derived from wild-type mice (24h post-infection). Each bar represents the mean ± SD of three independent experiments. **, $P < 0.01$.

To further demonstrate that this release is mainly TLR2 signaling dependent, TLR2 $^{-/-}$ MCs were infected with *E. faecalis in vitro*. As shown in FIG 3.5. the level of TNF-α and IL-6 released by TLR2 $^{-/-}$ MCs is significantly lower than the levels released by MCs from wild-type mice. The fact that the amount of cytokines released by TLR2 $^{-/-}$ cells is comparable to the amount released by MyD88 $^{-/-}$ cells argues for a central role of the TLR2 signaling pathway in the release of inflammatory cytokines by MCs in response to enterococci.

3.2.4.3. TLR2 signaling is essential for MC degranulation during *E. faecalis* infection

MC degranulation has been shown to be of critical importance for the antimicrobial properties of MCs to *E. faecalis*. To explore the contribution of TLR2 signaling in the recognition of *E. faecalis* by MCs, the degranulation response of MCs derived from mice deficient in the adaptor molecule MyD88 and mice deficient in the surface receptor TLR2 were studied in an *in vitro* infection with *E. faecalis*. MyD88 $^{-/-}$ as well as TLR2 $^{-/-}$ MCs showed a significantly lower potential in their ability to degranulate in response to *E. faecalis*, demonstrated by measuring the release of β-hexosaminidase – a lysosomal enzyme released by MCs which is used as a marker for degranulation [156] – in FIG 3.6. (A) in comparison to the corresponding

wild-type cells. This finding indicates once more the importance of the TLR/MyD88 signaling pathway in the recognition and global immune response of MCs towards this microorganism.

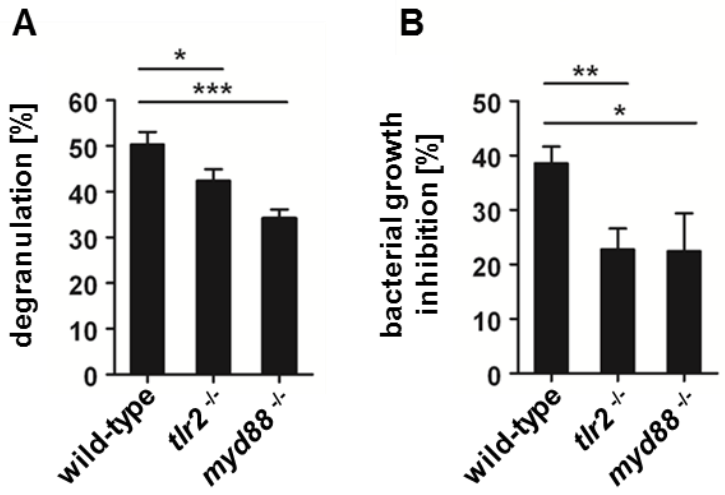

FIG 3.6.: TLR2 signaling is important for degranulation and control of *E. faecalis* growth. MCs derived from wild-type TLR2 $^{-/-}$ or MyD88 $^{-/-}$ mice have a lower ability to degranulate (A) and therefore to inhibit bacterial growth of *E. faecalis* (120 min post-infection) (B). Each bar represents the mean SD of quadruplicates from two independent experiments. *, $P < 0.05$; **, $P < 0.01$.; ***, $P < 0.001$.

Extracellular killing of enterococci by MCs are of central importance in immune defense of MCs against enterococci [158]. To determine the impact of TLR2-MyD88 signaling to the control of bacterial growth, MCs derived from MyD88 $^{-/-}$ and TLR2 $^{-/-}$ mice were infected *in vitro* with *E. faecalis* as described in section 2.12.1 and compared with MCs derived from wild-type mice. As depicted in FIG 3.6 (B) TLR2 $^{-/-}$ and MyD88 $^{-/-}$ MCs showed reduced degranulation that translates into reduced inhibition of the bacterial growth. These findings demonstrate the central importance of the TLR/MyD88 pathway in the immune response of MCs to *E. faecalis*.

3.2.5. Morphological change on bacterial surface of *E. faecalis* during co-cultivation with MCs

E. faecalis co-cultivated with supernatant of wild-type mast cells showed morphological surface changes (FIG 3.7. A and D). The formation of these vesicle-like structures on the

surface of *E. faecalis* was diminished by co-cultivated with supernatant from TLR2 $^{-/-}$ MCs (FIG 3.7. B) or medium control (C).

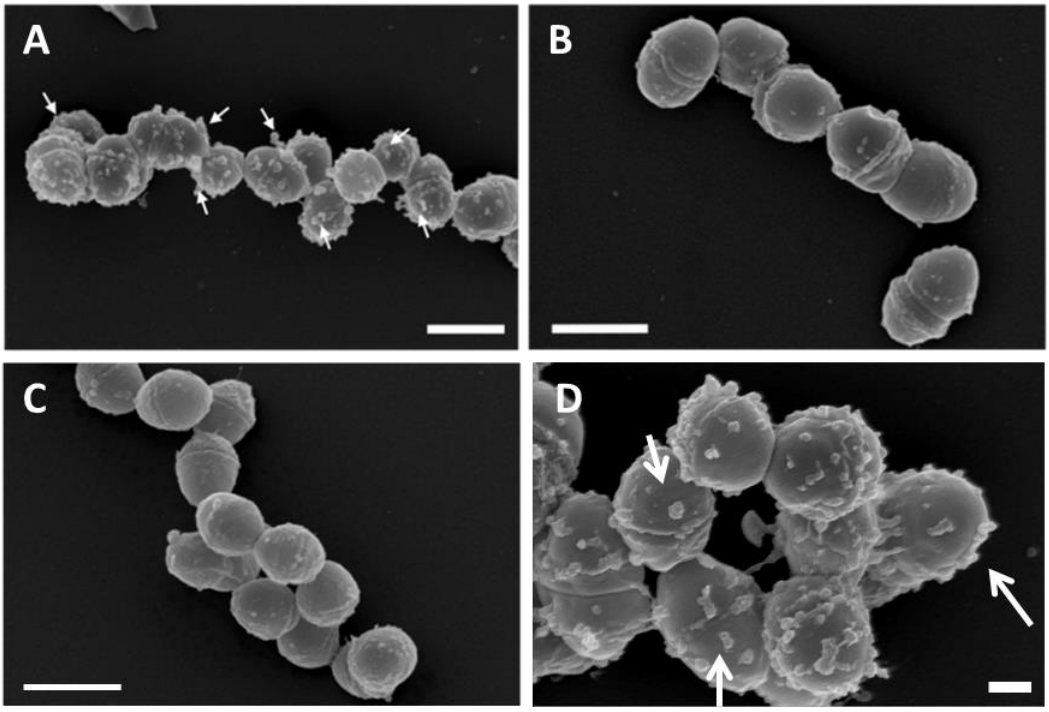

FIG 3.7.: SEM images of *E. faecalis* co-cultivated with either wild-type MCs (A and D), TLR2 $^{-/-}$ MCs (B), or medium alone (C) (white arrows indicate membrane vesicle formation in the surface of *E. faecalis*). Bar (A-C) represent 1µm, (D) 200nm. (SEM images by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research).

3.2.6. Cathelicidin LL-37 released by MCs in response to enterococci exhibits strong antimicrobial effects against these bacteria

MCs are able to release a variety of different components by degranulation [159]. One of these components is the cathelicidin LL-37, which is pre-stored in granules and immediately released after contact with degranulation-inducing stimuli. This antimicrobial peptide elicits a strong direct antimicrobial effect on Gram-positive bacteria [98, 99]. To investigate whether LL-37 is also of central importance in the ability of MCs to control bacterial growth of *E. faecalis*, the bacteria were treated with different concentrations of recombinant LL-37.

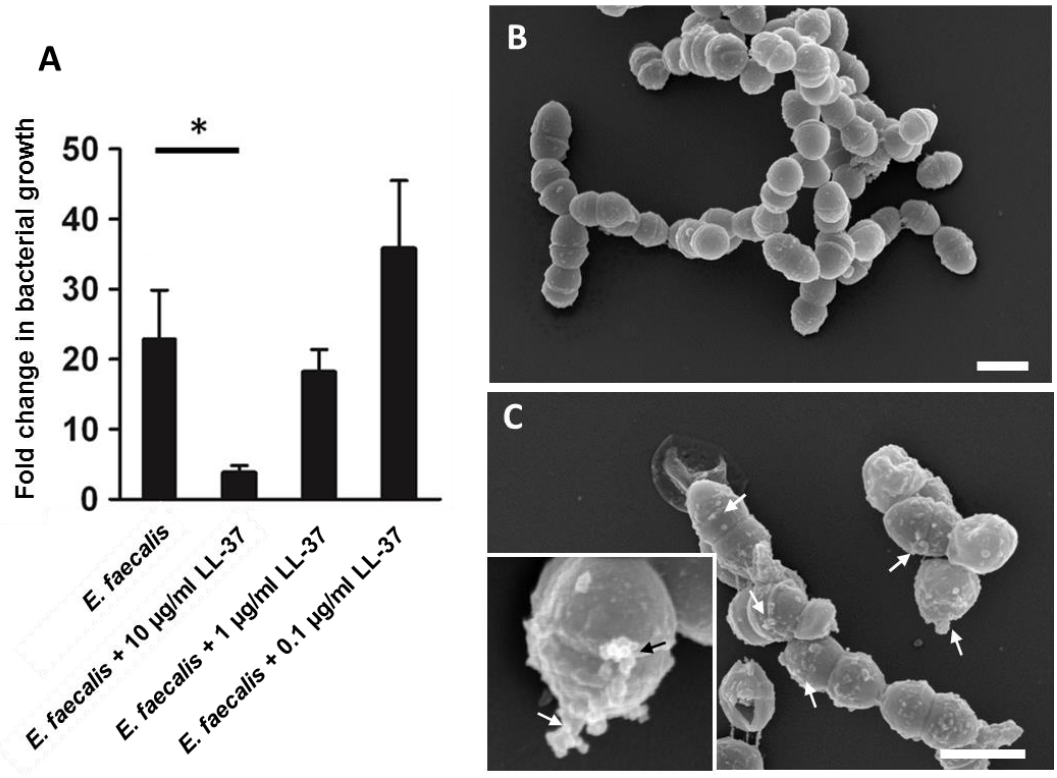

FIG 3.8.: LL-37 shows a potent antimicrobial effect on *E. faecalis.* (A) Different concentrations of LL-37 were added to a bacteria culture for 120 min and the bacterial growth was measured and compared to a medium control. 10 µg/ml LL-37 shows a significant higher bacterial growth inhibition compared to the control. The effect of LL-37 is dose-dependent. Each bar represents the mean ± SD of quadruplicates from independent experiments. *P <0.05. (C) Vesicle-like structures were observed when bacteria were treated with 10 µg/ml LL-37. These structures show a similar morphology compared to the membrane structures that have been observed when *E. faecalis* is co-cultured with wild-type MCs. Bacteria cultivated in medium alone serves as negative controls (B); bar represent 1µm. SEM images by Prof. Dr. Manfred Rohde, Helmholtz-Centre for Infection Research.

As demonstrated in FIG 3.8., LL-37 showed a potent dose-dependent antimicrobial effect against *E. faecalis.* Another interesting finding is that *E. faecalis* bacteria incubated in the presence of 10 µg/ml LL-37 showed similar surface associated vesicle-like modifications compared to bacteria that have been incubated within the supernatant of degranulated wild-type MCs (FIG 3.8. C and E). This finding reveals a potential role for LL-37 in the membrane destabilization processes, leading to bactericidal or bacteriostatic properties. Therefore, LL-37 is an essential antimicrobial component released during degranulation by MCs in response to these microorganisms.

3.3. Role of dendritic cells in *E. faecalis* infections

3.3.1. DCs are among the first immune cell populations entering the site of enterococcal infections

Dendritic cells are part of the first line of the innate immune defense against invading pathogens. DCs were found on sides that are exposed to the environment similar to macrophages or mast cells making them prime sentinels [103] for the recognition of microbial agents.

After recognizing microbes, DCs undergo a process called maturation. This process is characterized by upregulation of costimulatory markers and the release of inflammatory cytokines and chemokines [104]. Studies, which examine the interaction of enterococci with DCs, are very rare and their interaction with DCs is not intensively studied, even though DCs are important contributors of the innate immune response towards pathogenic bacteria.

The potential importance of DCs in the immune defense to *E. faecalis* is indicated by an intraperitoneal infection model of *E. faecalis* where DCs are among those innate immune cell types that significantly increased in number. The number of different invading immune cells was obtained by flow cytometry in mice, infected intraperitoneally with $5*10^8$ CFU of *E. faecalis* as described in 2.16.4.

Intraperitoneal infection of Balb/c mice with $5x10^8$ CFU of *E. faecalis* results in a massive increase of various immune cells like PMNs, macrophages and even MCs (FIG. 3.9. B-D). Interestingly, a marked and highly significant increase in the number of DCs could be observed following infection by *E. faecalis* compared to uninfected control mice (FIG. 3.9. A). These data suggest that DCs play an important role in coordination of host immune response towards enterococci.

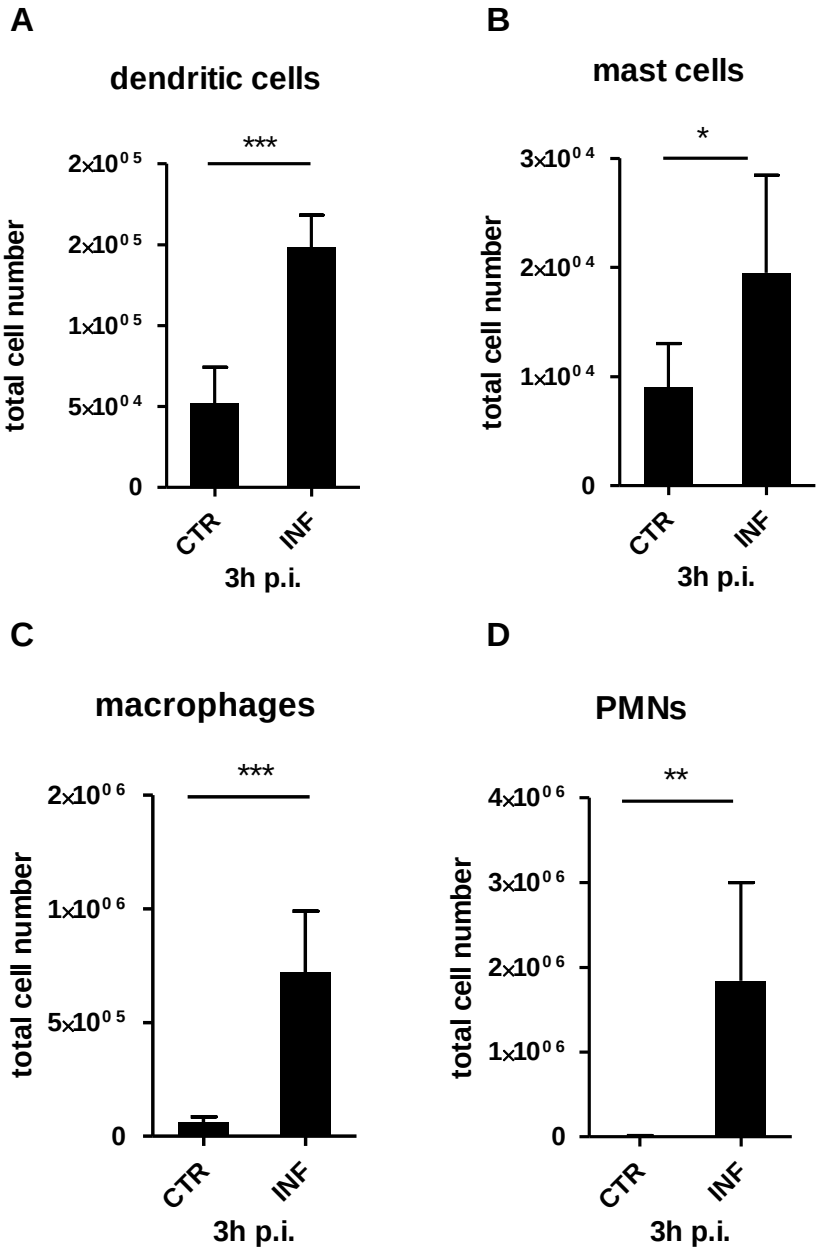

FIG 3.9.: Cellular response in the peritoneal cavity of Balb/c mice intraperitoneally infected with $5*10^8$ CFU of *E. faecalis*. Depicted is the infiltration of DCs (A), MCs (B), macrophages (C) and PMNs (D) into the peritoneal cavity of *E. faecalis* infected mice, 3h post infection (p.i.). Each bar represents the mean ± SD of five mice. *, $P < 0.05$; **, $P < 0.01$.; ***, $P < 0.001$.

3.3.2. *E. faecalis* is associated with DCs *in vivo*

To examine whether enterococci are associated with DCs *in vivo*, mice were injected intraperitoneally with $5x10^8$ CFU of GFP-expressing *E. faecalis* OG1RF bacteria or PBS as a control, and peritoneal washes were collected after 30 min. Peritoneal cells were stained with

PE-conjugated anti-CD11c antibodies and the proportion of CD11c^{high} GFP positive cells were analyzed by flow cytometry. As shown in FIG 3.9. upon administration with 5x10^{8} CFU, 20±4,1% of DCs were associated with GFP-expressing *E. faecalis*, the mean fluorescent intensity (MFI) increased from 478 (uninfected DCs) to 10567 (FIG 3.11.).

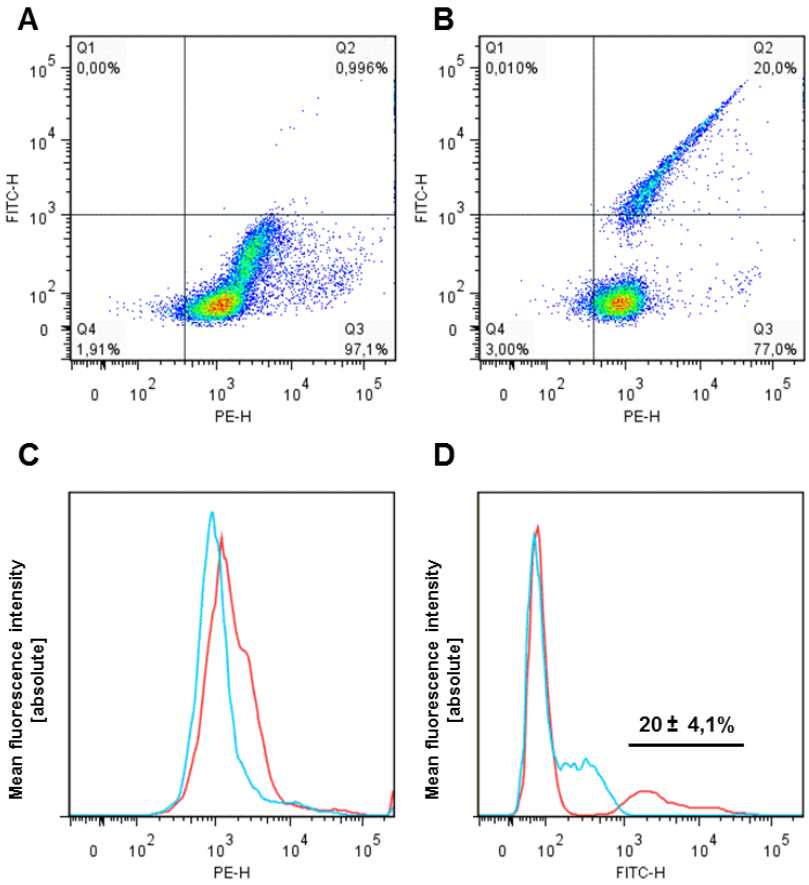

FIG 3.10.: Fluorescence panels showing co-localization of DCs and a GFP-expressing *E. faecalis* strain *in vivo*. The cells were collected as described in 2.16.4, counted and stained for flow-cytometry described in 2.14.3. (A) shows a quadrant-analyzation of DCs (PE $^{+}$) in a non-infected control (lower right quadrant). (B) shows the DCs (PE $^{+}$) colocalized with GFP-expressing *E. faecalis* (FITC^{+}) (upper right quadrant). (C) shows the MFI a histogram of CD11c of DCs infected with GFP-expressing *E. faecalis* (red) and the MFI of DCs (light blue) in a non-infected setting in PE channel. (D) shows a histogram of the DCs when DCs were colocalized with *E. faecalis* in red and in light blue the non-infected DCs in the FITC detection channel.

These data reveal a strong association of *E. faecalis* with DCs *in vivo* (FIG 3.10) underlining the importance of these cells in host innate immune response to enterococci. Methods applied are described in section 2.16.3.; 2.16.4. and 2.14.3.

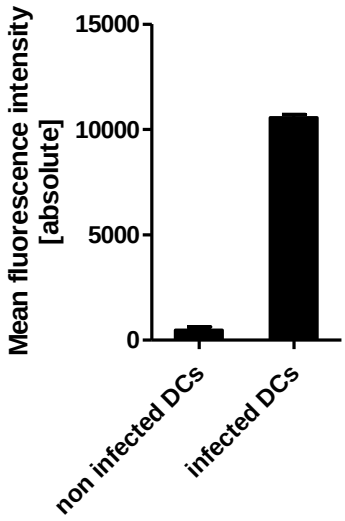

FIG 3.11.: Co-localization of DCs with *E. faecalis*. The increase in the GFP signal intensity indicates co-localization of DCs and GFP-expressing *E. faecalis in vivo*.

3.3.3. *E. faecalis*–induced expression of maturation markers on immature DCs

To investigate the ability of *E. faecalis* to induce DCs maturation, expression of maturation markers CD40, CD80 and CD86 on the cell surface were determined in DCs 24h after infection with *E. faecalis* by flow cytometry as described in section 2.14.3. The expression on unstimulated cells served as negative controls and stimulation with heat-killed *E. faecalis* bacteria were used as positive controls. The release of IL-12p70 after 24h post infection was also measured. DCs were infected as described in section 2.12.1 and the amount of cytokines released into the cellular supernatant was determined by ELISA as described in section 2.14.2. The results shown in FIG 3.12 indicate that infection with *E. faecalis* leads to efficient DC maturation as determined by upregulation of the surface marker CD40, CD80 and CD86 as well as the release of IL-12p70 during infection.

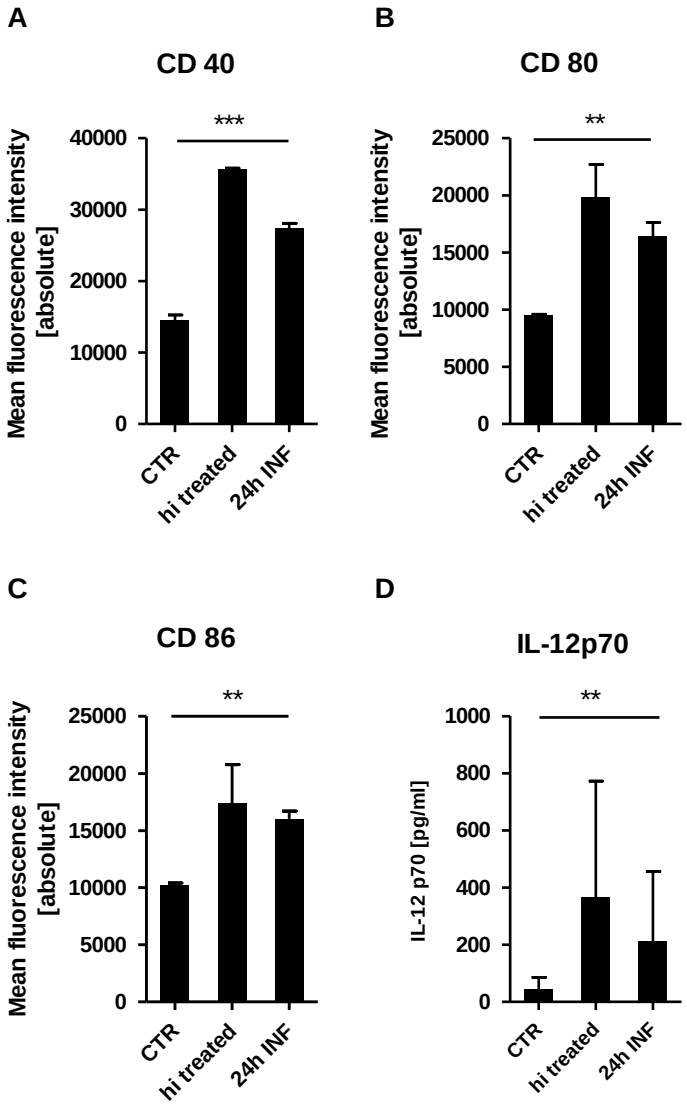

FIG 3.12.: Maturation of DCs after infection with *E. faecalis*. Maturation was determined by the upregulation of costimulatory proteins on the cellular surface of DCs. During *in vitro* infection of DCs with *E. faecalis* significantly higher levels of CD40, CD80 and CD86 are expressed on the cellular surface compared to a non-infected control (A and C). Furthermore the amount of IL-12p70 – the active form of IL-12 – is significantly higher compared to the levels released by non–infected control cells (D). One representative experiment out of four is shown. **, $P < 0.01$.; ***, $P < 0.001$.

3.3.4. DCs encountering *E. faecalis* elicit an inflammatory cytokine milieu

Next, it was examined which cytokines were released by DCs upon infection with *E. faecalis*. Bone marrow-derived DCs were infected as described in 2.12.1. Important immune regulatory effectors released by DCs are cytokines and chemokines involved in the modulation of the

humoral and cellular inflammatory response; therefore, the released levels of IFN-γ as well as the chemoattractant proteins KC and MIP2 were analyzed in the cellular supernatant of *E. faecalis* infected DCs.

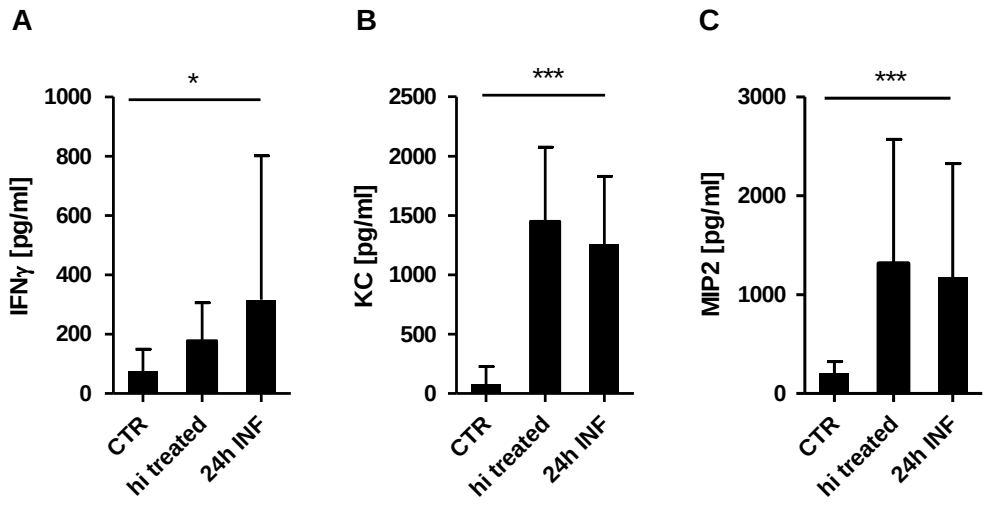

FIG 3.13.: Cytokine milieu of *E. faecalis* infected DCs. The release of proinflammatory cytokines und chemokines by DCs is increasing during infection with *E. faecalis*. Besides the potent immunomodulatory cytokine IFN-γ (A) the chemotactic active molecules KC (B) responsible for PMN recruitment to the site of infection and MIP2 (C) responsible for the recruitment of macrophages are induced by *E. faecalis* in DCs. Each bar represents the mean ± SD of five independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

The results, shown in FIG 3.13., indicate that DCs release several cytokines such as KC (B) and MIP2 (C) with immunomodulatory function in response to infections with *E. faecalis*, which have important chemotactic properties for the recruitment of phagocytic active cells to the site of infection, like PMNs and macrophages. These cells are additionally activated by IFN-γ, supporting a potential role of DCs in the coordination of phagocyte recruitment and activation during enterococcal infections.

3.3.5. *In vitro* phagocytosis of *E. faecalis* by bone marrow-derived dendritic cells (BMDC)

To determine the ability of DCs to phagocytize *E. faecalis* during *in vitro* infection, cells were infected with *E. faecalis* at an MOI of 10:1 as descript under 2.12.1. Briefly, after 2h the remaining extracellular bacteria were killed by incubation in the presence of 100 µg/ml of

gentamicin. At indicated time points, cells were washed twice with sterile PBS and lysed with 0.1% Triton X-100 in PBS. The number of viable bacteria was determined by serial dilutions plated on bile esculin agar plates.

The CFU enumeration showed that the number of surviving intracellular bacteria is significantly diminished over time; at 24h post infection nearly no viable intracellular bacteria are detectable (FIG 3.14.).

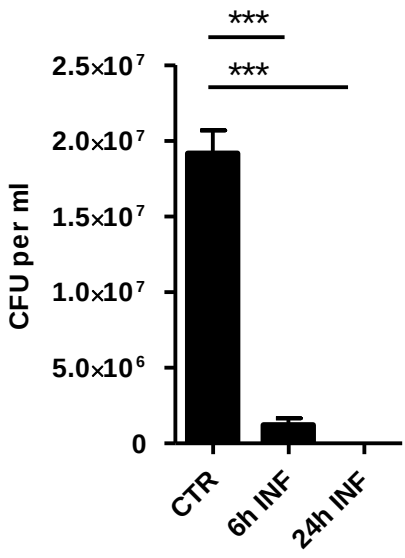

FIG 3.14.: Intracellular killing abilities of DCs against *E. faecalis*. DCs were infected as described in 2.12.1. Level of viable intracellular bacteria at 6 h and 24h post infection demonstrating an efficient killing of *E. faecalis* within DCs. ***, $P < 0.001$.

To confirm the killing of *E. faecalis* within DCs, fluorescence microscopy (FIG 3.15.) and transmission electron microscopy (FIG 3.16.) were applied. For double immune fluorescence studies, extracellular bacteria were labeled green and intracellular bacteria were labeled red. As shown in FIG 3.15. most of the microorganisms displayed red fluorescence, indicating that *E. faecalis* was very efficiently internalized by DCs.

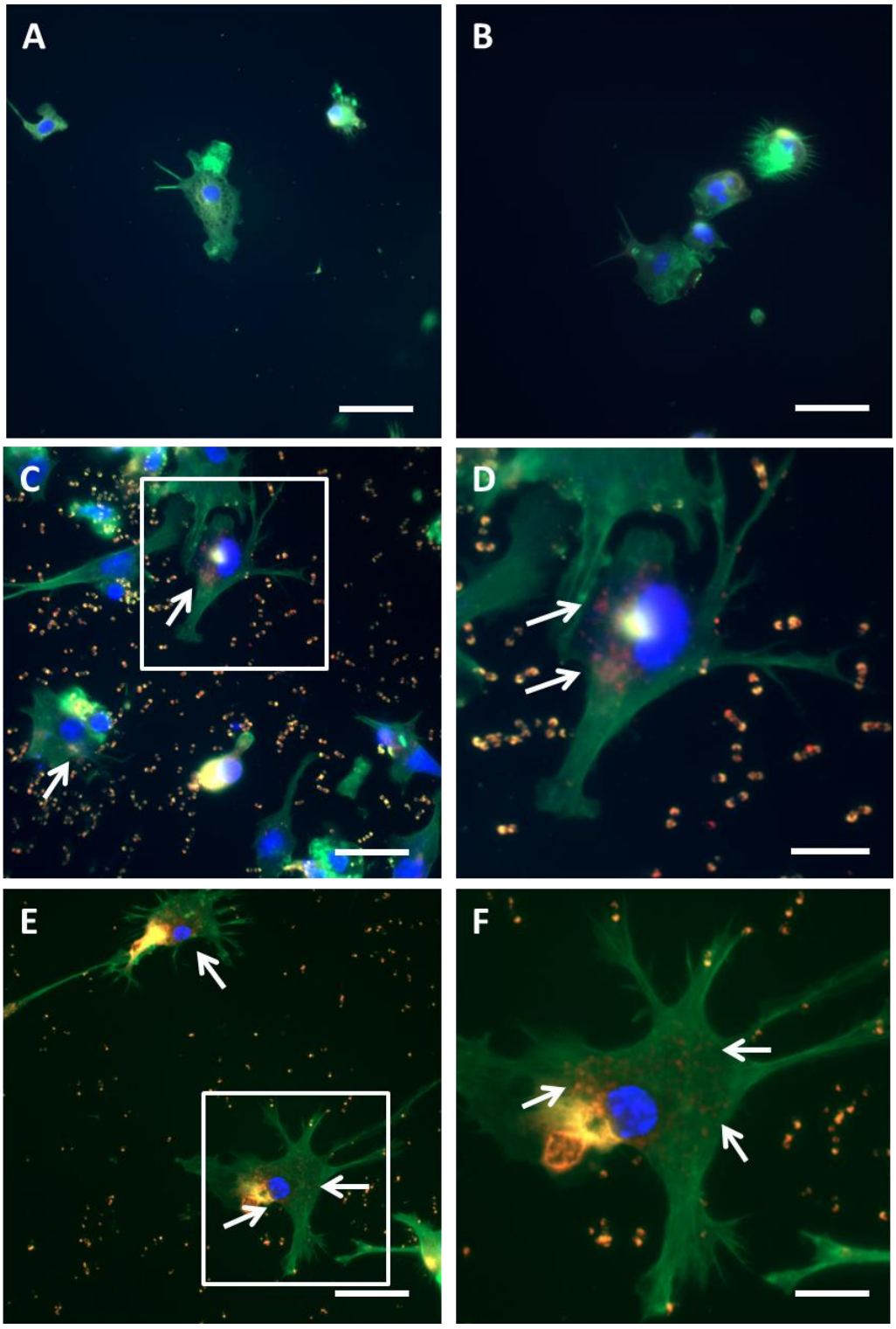

FIG 3.15.: Double-immunofluorescence image of DCs infected with *E. faecalis.* (A-B) non-infected DCs were stained as described in section 2.13.2. (C) DCs were infected with an MOI of 10:1 for 6h. Extracellular bacteria appear in yellow (merged channels of green and red) and intracellular in red. (D) Magnification of section (C). (E) DCs were infected with an MOI 10:1 of bacteria for 24h. (F) Magnification of section (E). Extracellular bacteria appear in yellow and

intracellular in red. Arrows mark intracellular bacteria. Bar represents (A-C; E) 10µm and (D; F) 1µm.

Transmission electron microscopic examination of *in vitro*-infected DCs revealed the presence of *E. faecalis* bacteria associated with and in the process of being phagocytosed by DCs (FIG 3.16. (B and D). Ultrathin sections of infected DCs displayed in FIG 3.16. revealed that *E. faecalis* resides intracellularly within phagosomes, where the bacteria undergo progressive degradation. FIG 3.16. shows that the degradation process of enterococci starts with the detachment of the capsule from the bacterial cell wall and the degradation of capsular material (indicated by white arrows in FIG 3.16. C).

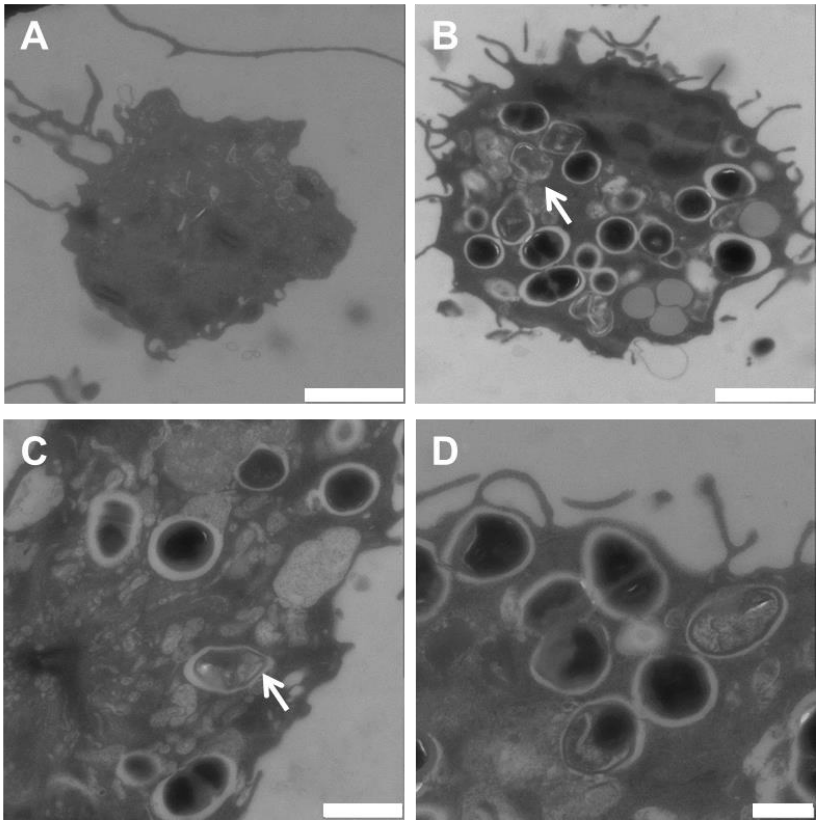

FIG 3.16.: Phagocytotic activities of DCs during infection with *E. faecalis*. DCs were infected as described in 2.12.1. The TEM images show (A) non-infected control cells and (B-D) phagocytic uptake of *E. faecalis* by DCs. Bar represent 2 µm (A-B), 1 µm (C) and 500 nm (D). TEM images were taken by Prof. Dr. Manfred Rohde, Helmholtz-Center for Infection Research.

3.3.6. *E. faecalis* is located and killed in lysosome-associated-membrane-protein-1 (LAMP-1) positive compartments within DCs

To gain further insights into the intracellular localization of ingested *E. faecalis*, it was investigated if ingested bacteria are localized within phagolysosome compartments using the marker LAMP-1. Therefore a LAMP-1 staining was performed using an Alexa568 conjugated anti-Lamp-1 antibody and a GFP-expressing *E. faecalis* strain (s. 2.13.2). FIG 3.17. (A) shows a non-infected control. FIG 3.17. (B) as well as the magnification of it in (C) show the co-localization of the GFP-expressing bacteria and the red LAMP-1 signal demonstrate the intracellular localization *E. faecalis* within LAMP-1 positive compartments. This observation is underlining a potent and highly efficient killing ability of DCs via lysosomal degradation of *E. faecalis*.

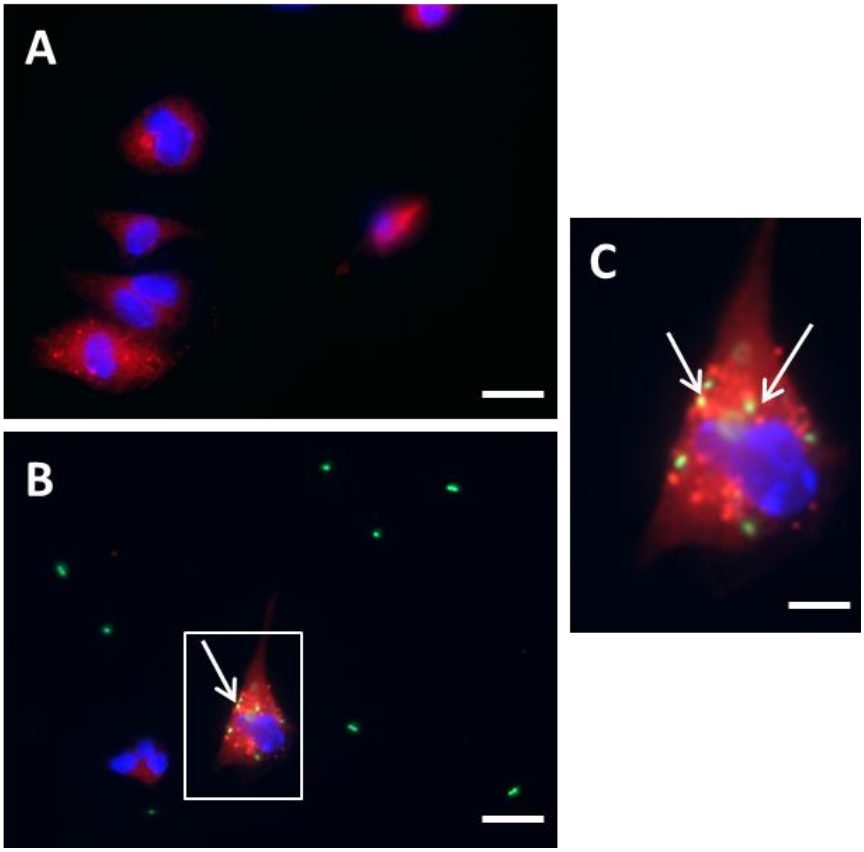

FIG 3.17.: *E. faecalis* is contained in LAMP-1 positive compartments within DCs. The immunofluorescence images of LAMP-1 positive compartments (red) show a co-localization with a GFP-expressing strain of *E. faecalis* (green), colocalized bacteria appear in yellow. (A) Non-infected control cells; (B) infection with a GFP-expressing strain of *E. faecalis*; (C) magnification

of (B). The DNA in the nucleus is counterstained with DAPI (blue). Bar represent A-B 10 µm, C 5 µm.

3.3.7. Effect of MyD88 deficiency on DC maturation

To determine the role of the MyD88 signaling pathway in *E. faecalis*-induced maturation of DCs it was investigated whether MyD88 is essential for *E. faecalis*-induced IL-12 production and release by DCs. Wild-type, TLR2 $^{-/-}$ and MyD88 $^{-/-}$ DCs were infected with *E. faecalis* for 2h and further cultured in the presence of 100 µg/ml penicillin/gentamicin. The supernatant of DCs stimulated with heat-killed bacteria was used as a positive control. The levels of IL-12p70 in the supernatants were measured 24h after infection by ELISA. As shown in FIG 3.18., secretion of IL-12p70 was abolished completely in MyD88 $^{-/-}$ DCs infected with *E. faecalis*, but only reduced moderately in TLR2 $^{-/-}$ derived DCs. These results indicate that *E. faecalis* induction of IL-12p70 is almost completely dependent on MyD88 signaling and independent of TLR2 indicating that other PRRs besides TLR2 play a role in this process.

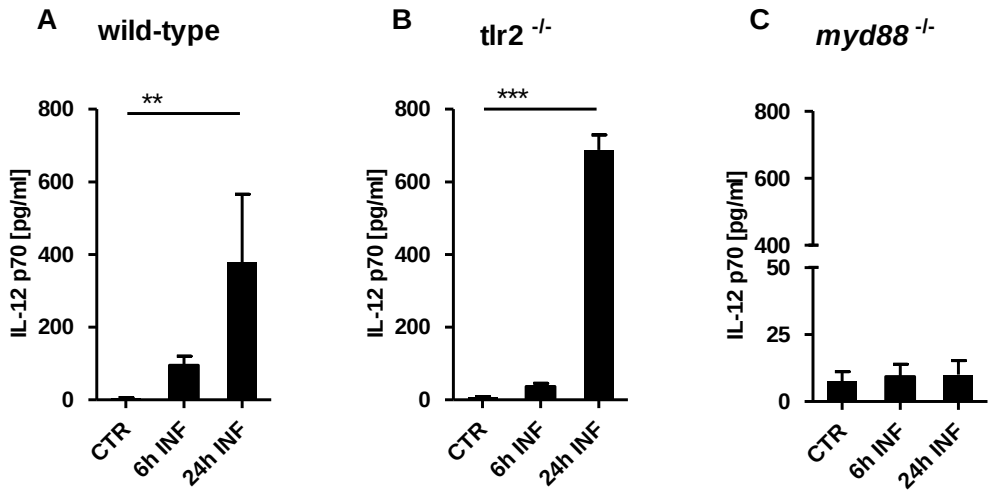

FIG 3.18.: DCs from TLR2 or MyD88 $^{-/-}$ mice release lower levels of IL-12 p70 than wild-type cells in response to *E. faecalis*. To identify the involvement of TLR2/MyD88 signaling in the maturation process of DCs during infections with *E. faecalis*, DCs derived from wild-type, TLR2 and MyD88 knockout mice were infected with an MOI of 10:1 of *E. faecalis* and the level of released IL-12 p70 was determined by ELISA. (A) wild-type cells, (B) TLR2 $^{-/-}$ and (C) MyD88 $^{-/-}$ DCs. **, $P < 0.01$; ***, $P < 0.001$.

3.3.8. Uptake and killing of internalized *E. faecalis* bacteria by DCs are independent of MyD88 expression

Cell activation by bacteria via TLR signaling regulates phagocytosis at multiple steps, including internalization and phagosome maturation [160-162]. Therefore, it was assessed whether DCs that lacked MyD88 compared to wild type DCs differ in their ability to internalize *E. faecalis* by counting the level of viable intracellular bacteria 24h post infection in bone marrow derived DCs. In contrast to the phenotype of these cells according to the induction of maturation marker and activation of inflammatory cytokine release, MyD88 expression did not affect the ability of DCs to internalize and kill *E. faecalis*, because the uptake of these bacteria by MyD88 $^{-/-}$ DCs (FIG 3.19. A) was comparable to the level of wild-type DCs.

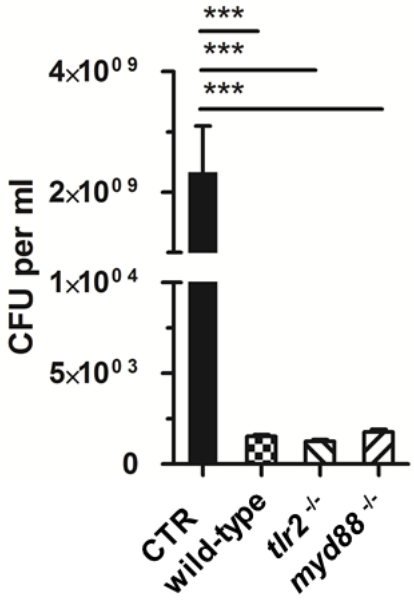

FIG 3.19.: DCs from TLR2 or MyD88 knockout mice have the same killing potential than wild-type cells. DCs from TLR2 or MyD88 knockout mice were infected as described in 2.12.2 and intracellular bacteria were counted 24h post infection. (A) No difference in the killing ability was observed among the different cell types. Each bar represents the mean ± SD of three independent experiments. ***, P <0.001.

3.3.9. Secretion of KC and MIP2 by DCs in response to *E. faecalis* is MyD88-dependent

Next, it was investigated if MyD88 was involved in the production of other inflammatory cytokines, such as KC and MIP2, these cytokines are shown to have an impact on the recruitment and infiltration of phagocytes to the site of infection [163]. These cytokines were detected by ELISA in the supernatants of uninfected and *E. faecalis* infected wild-type, TLR2 $^{-/-}$ or MyD88 $^{-/-}$ DCs 24h after *in vitro* infection. Wild-type DCs and MyD88 $^{-/-}$ DCs were

stimulated with heat-killed enterococci as controls. The production of both cytokines - KC (FIG 3.20. A and C) and MIP2 (FIG 3.20. D and F) - in response to *E. faecalis* infection was significantly reduced in MyD88 $^{-/-}$ DCs compared to wild-type cells. These data indicate that *E. faecalis*-induced production of proinflammatory cytokines in DCs is strongly TLR2 mediated and depends on MyD88 signaling.

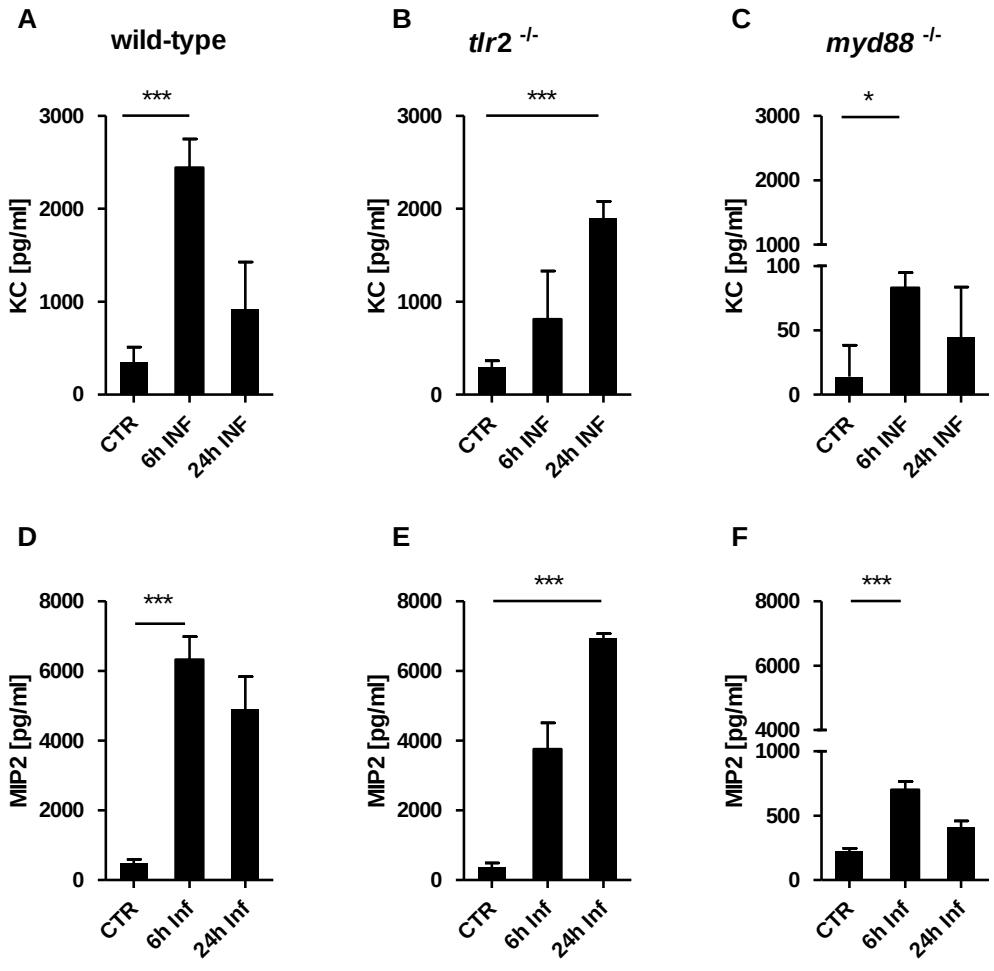

FIG 3.20.: KC and MIP2 release of DCs after infection with *E. faecalis*. DCs from TLR2 or MyD88 knockout mice were infected as described in section 2.12.1 and the amount of KC and MIP2 was measured. As shown in (A) DCs from wild-type mice release high levels of KC after 6h which is decreasing over time. (B) TLR2 $^{-/-}$ DCs showed a delayed release of KC peaking at 24h post-infection. (C) MyD88 $^{-/-}$ DCs are markedly impaired in their ability to release KC. MIP2 levels from DCs of wild-type mice (D), TLR2 $^{-/-}$ DCs (E) and MyD88 $^{-/-}$ cells (F) showed similar release patterns as described before for the chemokine KC. Each bar represents the mean ± SD of three independent experiments. *, P <0.05; **, P < 0.01; ***, P < 0.001.

In conclusion, these results indicate that immune recognition of *E. faecalis* by DCs is highly diverse and requires a coordinated interplay between MyD88-independent events involving phagocytosis as well as MyD88-dependent signaling pathways involved in the induction of DC maturation and cytokine production. The data further indicate an important role of DCs in the host defense towards this microorganism.

3.4. Interaction of *E. faecalis* with endothelial cells

The endothelium is a single cell thick barrier within the blood vessels, which separates blood from deeper tissue. ECs express surface markers like intercellular adhesion molecule 1 (ICAM-1 or CD54), vascular cell adhesion protein 1 (VCAM-1 or CD106) and mucosal addressin cell adhesion molecule 1 (MA*d*CAM-1) which are necessary for the migration process of leukocytes through the endothelium [128]. Endothelial cells are also important immune modulators with various regulatory properties on other branches of the immune system [129]. Furthermore, they provide anti-pathogenic functions through the release of antimicrobial peptides [145].

E. faecalis is able to induce endocarditis by establishing a biofilm on the heart bulb or in middle heart blood vessels. Because the endothelial barrier is the first line of the host innate immune response encountering enterococci when they enter the blood stream, the interaction with these cells is of central interest. To better understand the colonization of the endothelium in disease, it is necessary to unravel the molecular and cellular processes taking place during the interaction of enterococci with endothelial cells.

3.4.1. *E. faecalis* is able to adhere to but does not internalize into endothelial cells

Many pathogens use intracellular compartments to protect themselves from cells of the host immune defense like PMNs or macrophages. A few studies indicated an intracellular stage for *E. faecalis* [164]. The internalization into eukaryotic cells is a process that can be initiated by pathogens through active invasion or by phagocytosis of host cells [165].

To address this question, HUVECs were infected as described in section 2.12.2. After 2h the cells were washed twice with 1x PBS to remove extracellular bacteria and were cultivated further for up to 22h in the present of 100 µg/ml penicillin/streptomycin and 100 µg/ml gentamicin. Samples of infected HUVECs were taken after four, seven and 24h post-infection. HUVECs were washed twice with 1x PBS to remove extracellular antibiotics. Then the cells were lysed with 0.1% Triton X-100 and intracellular bacteria were plated on BEA

agar plates. The numbers of viable, intracellular *E. faecalis* within HUVECs were counted. Interestingly, almost no intracellular bacteria could be detected. Only one cell out of 200 showed an intracellular presence of bacteria 4h post infection. Low levels of intracellular bacteria were detectable up to 24h post-infection.

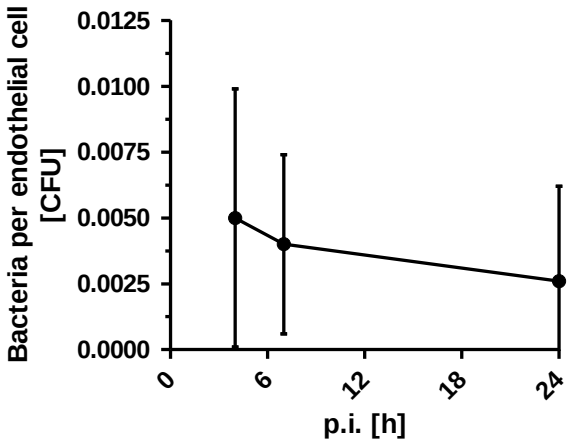

FIG. 3.21.: *E. faecalis* shows very low frequency of intracellular bacterial presence within HUVECs. Endothelial cells were infected with an MOI of 10:1 bacteria per cell as described in section 2.16.2. The numbers of intracellular bacteria were counted after 4h, 7h and 24h post infection (p.i.) by plating live bacteria on BEA-plates in serial dilutions. The line represents the mean ± SD of three independent experiments.

To strengthen these findings double immunofluorescence microscopy was applied. HUVECs were infected with live bacteria at an MOI of 10:1 bacteria per cell as described in 2.12.2 and stained as described in 2.13.2. The intracellular bacteria appear in red, extracellular bacteria appear in yellow. A representative picture is depicted in FIG. 3.22. (A and B). Almost no intracellular bacteria were found within the endothelial cells. These data support the counting of viable intracellular bacteria obtained by plating serial dilutions of lysed endothelial cells.

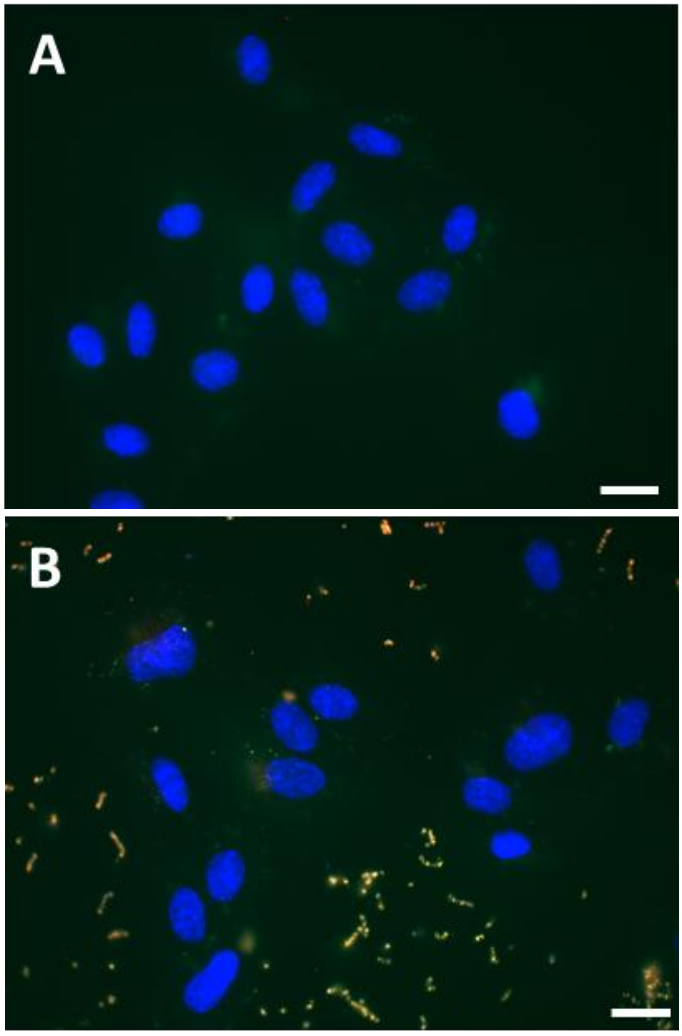

FIG. 3.22.: Representative double immunofluorescence image of HUVECs infected with *E. faecalis.* Endothelial cells were infected with an MOI of 10:1 bacteria per cell as described in section 2.16.2., stained as described in section 2.13.2. (A) Non-infected control. (B) *E. faecalis* infected HUVECs 24h post-infection showing no presence of intracellular bacterial within endothelial cells. One representative image out of ten experiments is shown. Bar in A and B represents 50 µm.

3.4.2. Cytokines involved in the chemotaxis of neutrophil recruitment are manipulated by *E. faecalis*

As a main source for cytokine and chemokine release endothelial cells play a crucial role in the recruitment of innate immune cells [130-133]. One of these cytokines is the proinflammatory cytokine IL-8 which is the most predominant attractant for the recruitment of polymorphonuclear neutrophils (PMNs) to the site of infection [163]. To obtain the cytokine profile in the course of *E. faecalis* infections, HUVECs were co-cultivated with *E.*

faecalis as described in section 2.12.2 with an MOI of 10:1. 2h after infection a mixture of 100 µg/ml penicillin/streptomycin/gentamicin was added into the cell culture to kill extracellular bacteria. The cells were incubated further for 22h. After 24h of total infection time, the supernatant was collected and an ELISA to detect the released levels of IL-8 was performed as described in section 2.14.2.

The amount of IL-8 determined in the cell culture supernatant of *E. faecalis* infected cells reached the level of uninfected control cells, whereas heat-killed *E. faecalis* induced a significant increase in the release of IL-8 by endothelial cells (FIG. 3.23.).

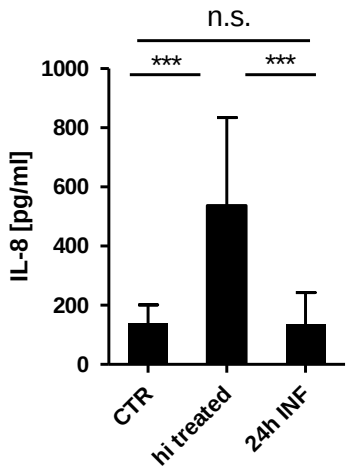

FIG. 3.23.: IL-8 released by HUVECs after infection with *E. faecalis*. During infection with live bacteria (described in section 2.12.2) no release of the proinflammatory cytokine IL-8 by HUVECs could be detected, whereas heat inactivated enterococci are able to mount a significant IL-8 release by HUVECs *E. faecalis.* Each bar represents the mean ± SD of four independent experiments. ***, $P < 0.001$.

The diminished response of endothelial cells during infection with live *E. faecalis* bacteria compared to the response elicited by heat-killed microorganisms indicated that actively produced and released factors of *E. faecalis* are responsible for the down-regulation of IL-8 secretion by endothelial cells after they have encountered the bacteria.

3.4.3. No cytotoxicity on endothelial cells by *E. faecalis* infection

To investigate the role of *E. faecalis*-induced cytotoxicity on endothelial cells that could interfere with the observed diminished IL-8 response, the release of lactate dehydrogenase

(LDH), an enzyme associated with necrotic cell death, was determined. LDH serves as indicator of necrotic cell death caused by an external influence or trigger [166, 167].

Briefly, HUVECs were infected as described in section 2.12.2. with *E. faecalis*. No LDH release was detectable in cells infected with enterococci suggesting absolutely no cytotoxic effect on endothelial cells.

3.4.4. *E. faecalis* manipulates the expression of the endothelial surface receptor involved in leucocyte extravasation ICAM-1 during infection

HUVECs cells recruit PMNs via release of IL-8. The expression of endothelial surface-associated receptors like ICAM-1 (CD54), E-selectin (CD62-E) and VCAM-1 (CD106) promotes rolling of PMNs and the extravasation of PMNs into infected tissue. The expression and the availability of these receptors are critical for clearance of bacterial infections.

To investigate the influence of *E. faecalis* infection on the expression of these surface markers, HUVECs were infected with *E. faecalis* at an MOI of 10:1 as described in 2.12.2. Because IL-1β is one of the most prominent inducers for ICAM-1 expression [168], endothelial cells were stimulated with recombinant IL-1β at 1 ng/ml as a positive control (FIG. 3.24.).

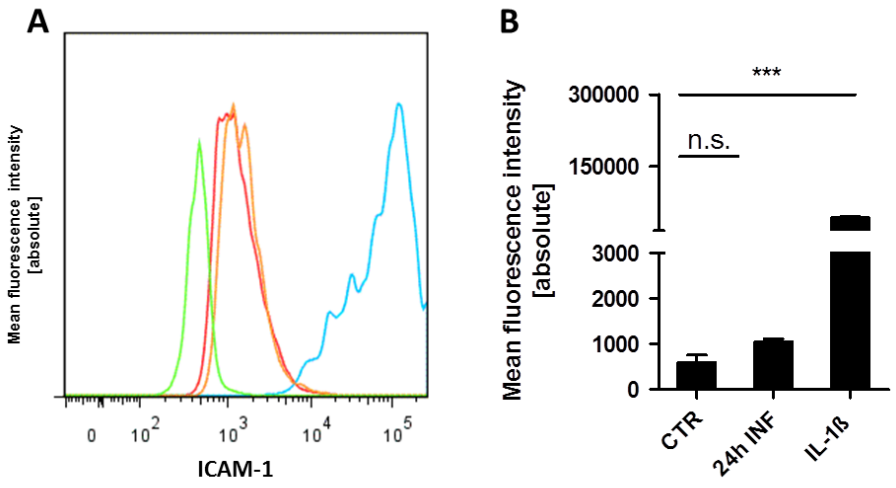

FIG. 3.24.: Flow cytometry analysis of ICAM-1 (CD54) surface expression after infection with *E. faecalis*. 5x10^4 HUVECs were infected with an MOI of 10:1 with *E. faecalis* and analyzed by flow cytometry. (A) The presence of the surface receptor CD54 was analyzed and quantified by the mean fluorescence intensity. The expression during infection with enterococci (orange) was compared to a non-infected control (red) and HUVECs treated with recombinant IL-1β as positive control (light blue). Unstained control cells are represented in green. One representative result out

of three is shown. (B) MFI of CD54 surface expression was calculated on *E. faecalis* infected cells. Each bar represents the mean ± SD of three independent experiments. ***, P < 0.001.

This finding together with the moderate induction of the chemotactic cytokine IL-8 underlines the possibility that *E. faecalis* has evolved strategies to interfere with the inflammatory response of the endothelium (FIG. 3.24.) on the level of cytokine release and chemoattraction of phagocytes.

3.4.5. *E. faecalis* is also affecting the expression of E-Selectin and VCAM-1 on the surface of endothelial cells

Leukocyte extravasation is not only based on the expression of surface receptor ICAM-1, but also on the expression of E-selectin and VCAM-1. To better understand how *E. faecalis* manipulates the extravasation process of leukocytes, the surface expression of E-selectin and VCAM-1 in response to *E. faecalis* was also analyzed (FIG. 3.25.). As already demonstrated for ICAM-1 (FIG 3.25. A), *E. faecalis* induces only a moderate expression of the extravasation receptors E-selectin (B) and VCAM-1 (C) compared to IL-1β-induced expression of these receptors. These findings indicate that *E. faecalis* is able to hinder the surface expression of extravasation receptors on endothelial cells and therefore, the recruitment of inflammatory cells (*e.g.* PMNs) to the site of infection.

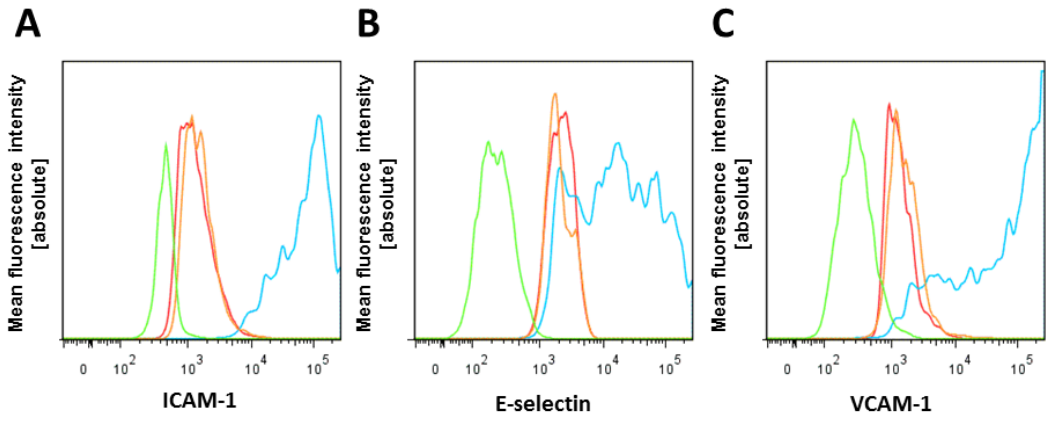

FIG. 3.25.: Surface expression of ICAM-1; E-selectin and VCAM-1 on endothelial cells during infection with *E. faecalis*. 5x10^4 HUVECs were infected with an MOI 10:1 of *E. faecalis* and analyzed by flow cytometry. All extravasation receptors, ICAM-1 (A), E-selectin (B) and VCAM-1 (C) show only moderate surface presence after stimulation with *E. faecalis* compared to IL-1β stimulated cells which served as positive control. Infected cells (orange) were compared to a non-

infected control (red) and an IL-1β treated positive controls (light blue); an unstained control is shown in green. One representative result out of three is shown.

3.4.6. Expression of leukocyte extravasation receptors on endothelial cells after infection with *E. faecalis* is independent on host genetics and donor specificity

To investigate the influence of host genetics and cell variability on *E. faecalis* induced extravasation receptor expression, HUVECs of different origins representing the diversity of human donors were used in co-cultivation studies.

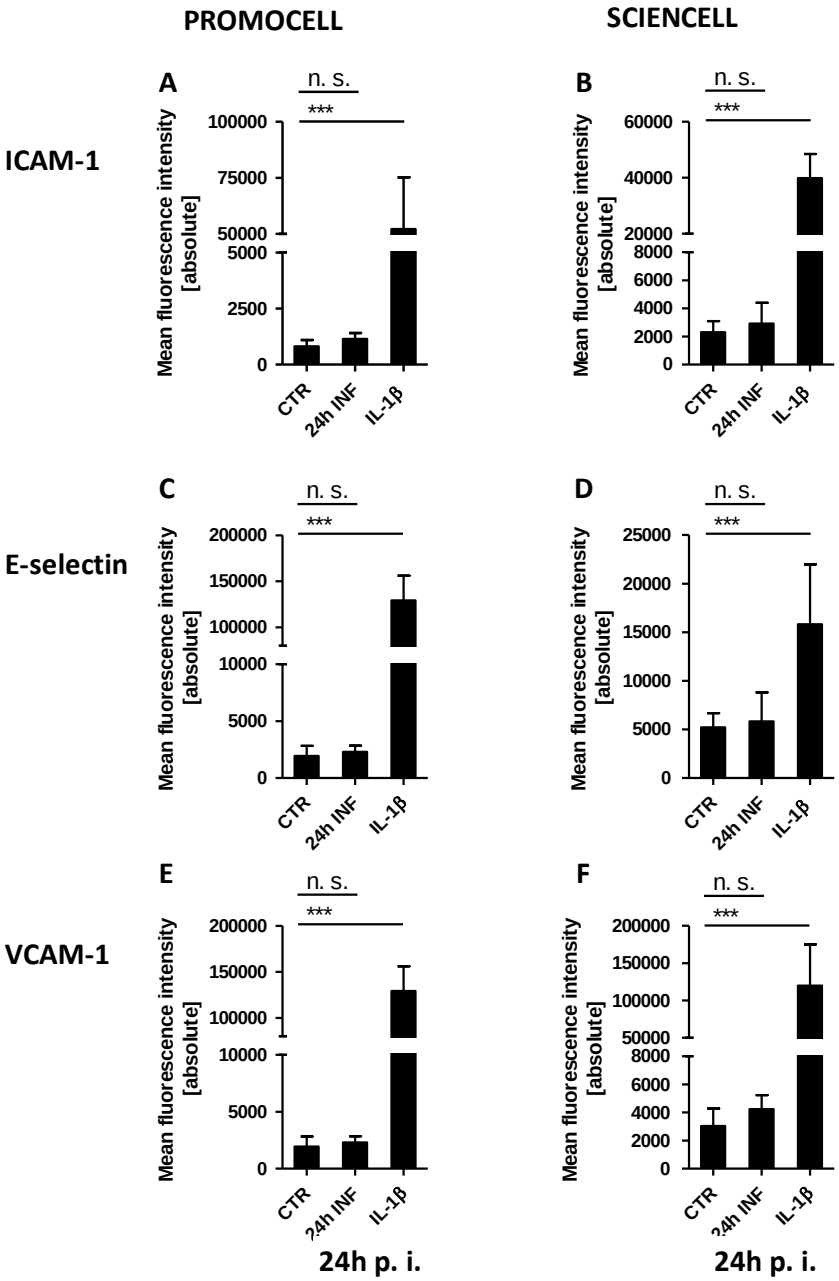

FIG. 3.26.: Surface expression of ICAM-1, E-selectin and VCAM-1 on HUVECs of different origins, representing a unique donor spectrum after co-cultivation with *E. faecalis*. HUVECs were

infected as described in section 2.12.2 for 24h with an MOI of 10:1 with bacteria and the cells were analyzed by flow cytometry as described in section 2.14.3. Right panel (A; C; E) shows the response of HUVECs from PROMOCELL; left panel (B; D; F) the response of HUVECs from SCIENCELL. Each bar represents the mean ± SD of three independent experiments.

Briefly, HUVECs (PROMOCELL, SCIENCELL) were co-infected with *E. faecalis* at an MOI of 10:1 as described in section 2.12.2. After 24h the cells were stained for ICAM-1, E-selectin and VCAM-1 and analyzed by flow cytometry. The mean fluorescent intensity (MFI), obtained by flow cytometry analysis is depicted in FIG 3.26.

No difference was observed in the surface presence of ICAM-1, E-selectin and VCAM-1 on endothelial cells from two origins. These findings support an enterococcal component based and host independent characteristic of the observed weakening effect of endothelial extravasation marker expression during infection with *E. faecalis*.

3.4.7. Comparison of activation pattern between *E. faecalis, S. aureus* and *E. coli* stimulated HUVECs

To further confirm the ability of enterococci to manipulate the expression of endothelial surface receptors involved in the extravasation process of leukocytes, in comparison to bacteria known to induce the expression of these receptor [169-171] the response of HUVECs to infection with *S. aureus* and *E. coli* was investigated for the expression of ICAM-1, E-selectin and VCAM-1 compared to *E. faecalis*.

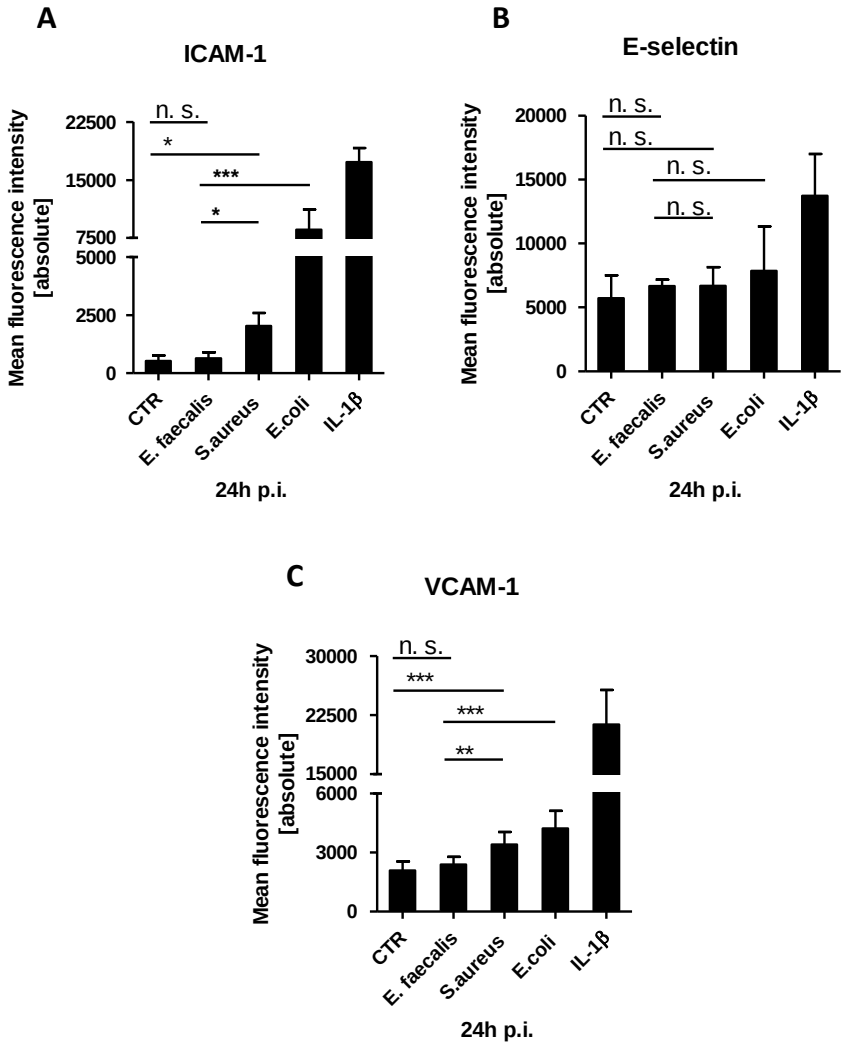

FIG 3.27.: Surface expression of ICAM-1, E-selectin and VCAM-1 on HUVECs stimulated with *E. faecalis*, *S. aureus* or *E. coli*. The difference in the expression of these surface receptors was detected by flow cytometric analysis ICAM-1 as well as VCAM-1 are significant higher expressed in cells infected with *S. aureus* or *E. coli* compared to non-infected control cells. HUVECs infected with *E. faecalis* do not show upregulation of these surface receptors compared to the uninfected control cells. Interestingly the expression of the E-selectin was not significantly different under all infection conditions compared to non-infected cells. Each bar represents the mean ± SD of three independent experiments. ****, $P < 0.0001$.

These data underline that in comparison to other bacterial pathogens like *S. aureus* or opportunistic organisms like *E. coli*., *E. faecalis* manipulates the expression of surface receptors responsible for leukocyte extravasation for its own benefit. This indicates an immune evasion strategy in which the docking and therefore the infiltration of neutrophils through the endothelial barrier is blocked.

Nevertheless, further experiments will have to be performed to unravel the underlining molecular processes involved in this silencing effect that *E. faecalis* is able to induce on the endothelial barrier.

4. Discussion

Enterococcus faecalis is the main cause of enterococcal bacteremia and nosocomial bloodstream infections in the hospital environment [11]. Up to 90% of enterococcal infections in humans are caused by the opportunistic pathogen *E. faecalis* [172]. Enterococcus diseases are meanwhile among the most common reasons for nosocomial infections in the US and Europe. About 12% of all nosocomial infections in the US and 26.5% of urinary tract infections in Germany for example are caused by this pathogen [16, 17]. The increasing problems of life-threatening infections induced by this organism demonstrate the need for intensive investigation on host pathogen interactions during enterococcal infections.

Most virulence studies and infections of enterococci have been performed using *E. faecalis* strains. This enterococcus species is not only responsible for most of the enterococcal infections, its virulence abilities are also less difficult to manipulate compared to other enterococcal species like *E. faecium*. Nevertheless, the secreted factors, as well as the surface localized virulence properties, which have been found to be involved in the severity of infection caused by this bacterial family is very similar between both bacterial species. Since the beginning of the antibiotic era these bacteria are a challenge for the medical field because of their multiple variants of antibiotic resistances [24, 25]. The need for adaptive combinations of antibiotics to successfully treat severe enterococcal infections, like infective endocarditis is therefore increasing [10].

For blood stream infection and sepsis *E. faecalis* has become a serious issue in North America and in Europe [19, 21]. Furthermore there are also case-reports of infection of the respiratory tract, osteomyelitis, cellulitis and infection of the central nervous system and even meningitis [22, 23], reported to be causally determined to enterococci underlining the broad spectrum of diseases that can be provoked by this opportunistic microorganism.

The increased incidences of enterococcal infections in Germany [173] have not been linked to a remarkable variability in virulence or severity so far [174]. Nevertheless, the reasons for the change in the epidemiology and clinical manifestations of enterococcal infections are still unknown. An area of intensive investigation remains whether this dramatic rise in the incidence of severe infections caused by enterococci resulted from changes in virulence characteristics or if this phenomenon is associated with increased susceptibility of the host presumably because there is a lack in mounting a protective immune response towards these bacteria. All possibilities do not seem to be exclusive, and there is little doubt that the disease outcome is also strongly determined by the host-pathogen interplay.

Therefore, one of the initial steps in this project was to screen a collection of well-characterized clinical isolates for the presence/absence of virulence factors known to be involved in the pathogenesis of enterococcus diseases and to search for correlations between disease manifestations on one side and the genetic basis of the bacteria on the other side.

The main virulence factors of *E. faecalis* are genes located on a 153 kb pathogenicity island (PAI) of these bacteria [32, 33]. The majority of these genes are responsible to enhance the colonization potential of enterococci but also cytotoxins or stress response proteins are encoded in the PAI region.

In this study the genes for adhesion of collagen binding by *E. faecalis* (*ace*), aggregation substance (*as*), cytolysin A (*cylA*), which is part of the cytolysin activation process, enterolysin A (*elyA*), enterococcal surface protein (*esp*), endocarditis and biofilm-associated pilus (*ebpABC*), endocarditis antigen (*efaA*), gelatinase E (*gelE*) and hyaluronidase (*hyl*) were analyzed with respect to the disease manifestation of the corresponding strain.

Additionally, the ability of clinical isolates to completely lyse erythrocytes (β-hemolysis) was tested, but could not be connected to the expression of the two known cytolysins CylA or ElyA. The discrepancy of the presence of the coding gene and the protein might be due to the complexity of the machinery responsible for the release of cytolysins. Cytolysin consists of two subunits called CylL" and CylS". These two subunits were modified by an extracellular protease called CylA. Without this modification, the two subunits are not able to establish a pore within the membrane of host cells [37, 175]. In this study not all of the related genes involved in the production and modification of a full functional protein cluster were investigated which could be the reason for the gap between the presence of the gene *cylA* and the lack of β-hemolysis activity. A summary of the results is depicted in the appendix (table 10).

In this study no correlation could be found between the genotype and disease manifestation. The imbalance between enterococcal virulence attributes and specific host immune defense properties is highly likely the reason for the ability of *E. faecalis* to colonize, invade and damage host tissue. However, the molecular and evolutionary mechanism leading to this imbalance is still not clear. Defining virulence mechanisms employed by *E. faecalis* and characteristics of the host that contribute to pathogenesis will facilitate the development of both pharmacological and immunological therapies.

Despite the fact that *E. faecalis* is an emerging nosocomial pathogen causing increasing problems not only in ICUs or in immunocompromised patients, but also for patients undergoing surgery, the knowledge of how this pathogen is interacting with the host immune

system and which processes are involved in the onset, progression and pathogenesis of diseases caused by *E. faecalis* is still extremely limited. Only a few studies have attempted to investigate this area systematically. This knowledge, however, could lead to new strategies of how to improve the natural host resistance to this emerging pathogen. Investigations to unravel the interaction of enterococci with host immune cells have been strongly focused on the role of professional phagocytic cells so far. In this regard, several reports have highlighted a crucial role of PMNs in the early control of *E. faecalis* [55]. It has been shown that opsonization of *E. faecalis* and killing by PMNs play a central role in the host immune response to enterococci [55]. Also the translocation of *E. faecalis* in mice, leading to sepsis was shown to be dependent on functional PMNs [56]. The findings in this work also argue for a potential intracellular survival of enterococci within phagocytic cells.

A factor responsible for the intracellular survival in macrophages seems to be PerA [57]. According to Zou and Shankar PerA inhibits the activation of the lysosomes and the fusion of lysosomes and endosomes, enabling the bacteria to survive within macrophages for a long time period intracellularly [58].

Therefore, one aim of this study was to gain deeper mechanistic insight into the interaction of *E. faecalis* with cells of the host immune system, especially focusing on innate immune cells that have not been investigated in great detail. In addition to phagocytes, various other immune cell types are of pivotal importance in the coordination of an effective immune response. Sentinel cells composing the first line of immune defense against invading pathogens are mast cells, dendritic cells or in particular cells building up the endothelial barrier. The interaction and meaning of these cells during infections caused by enterococci is not a strong focus of research up to now or the results are conflicting and contradictory.

So far, sentinel cell types underestimated in the context of infectious disease *per se* are MCs [84, 93, 176], which have been linked and intensively investigated for their role during allergic reactions. Nonetheless, the interest on the antimicrobial properties of these cells is increasing. MCs are inflammatory cells, which are typically located immediately beneath the epithelial surfaces exposed to the outer environment such as the skin and the mucosa, but also the respiratory system, genitourinary and gastrointestinal tract [94]. Because many of these sites are also common entry ports for pathogens, MCs represent one of the first inflammatory immune cells encountered by an invading microorganism and initiating the host immune response against these microbes [177]. Recent evidences have suggested that MCs have a beneficial contribution to both innate and adaptive immunity during infection [178], because

MCs are able to release pre-stored and newly synthesized inflammatory mediators as well as antimicrobial acting substances into their surrounding upon contact with pathogens [93, 94]. In this study, an in-depth characterization of the interactions of *E. faecalis* with MCs was performed.

The results demonstrated that *E. faecalis* is not only able to adhere to MCs, but also to stimulate an inflammatory cytokine response by the release of IL-6 or TNF-α. IL-6 release by MCs was recently shown to be important to regulate the selective influx of other immune cells like dendritic cells (DCs) into inflamed lymph nodes, thus enhancing opportunities for effective T-cell-DC interaction. These interactions induce an effective adaptive immune response to bacterial pathogens [85]. In particular, the release of TNF-α by local MCs are an important protective response elicited by MCs to enterococcal infection, because TNF-α serve as a central chemoattractant for phagocytes, like PMNs and macrophages, important for elimination of this pathogen [56, 179]. Nevertheless, also an intracellular persistence of *E. faecalis* in macrophages has been reported [57, 164, 180] illustrating that *E. faecalis* has also evolved strategies to manipulate host immune defense mechanism.

Interestingly, no evidence could be found in this study that *E. faecalis* is able to actively invade or even persist in great numbers within MCs. This is in contrast to other Gram-positive pathogens like *Staphylococcus aureus* [90] or Gram-negative bacteria like *Escherichia coli* [181]. These bacteria gain access into mast cells without the loss of bacterial viability by a route distinct from the classical endosome-lysosome pathway and are even able to persist for long time periods within MCs. If MCs were infected with opsonized heat-killed *Salmonella typhimurium* or other opsonized enterobacteria, an efficient phagocytosis of these organisms was observed underlining the phagocytic properties of these cells [95, 96].

MCs have been shown to be able to kill bacteria by two different mechanisms [89, 90, 97, 182]: (1) phagocytosis (mainly Gram-negative bacteria) and/or (2) extracellular by degranulation and the release of antimicrobial peptides such as LL-37, which affects several bacteria, like *S. aureus* [90] or by the formation of so called mast cells extracellular traps (MCETs) which has been shown to be important in the efficient killing of *S. pyogenes* [97]. This study could also prove that MCs were activated after encounter of *E. faecalis* and exerted a direct antimicrobial activity against this pathogen via several extracellular mechanisms.

One of those mechanisms involves the formation of extracellular traps. Immunofluorescence microscopical studies revealed that *E. faecalis* is able to induce a certain level of MCETs formation and could also show that at least some of the entrapped bacteria were killed within the MCET structure [158]. The level of MCET formations was not as pronounced as it has

been reported for other pathogens like *S. pyogenes* [97]. Treatment with nucleases also did not completely abolish the antimicrobial function of MCETs. This leads to the conclusion that entrapment by MCETs, which are produced by infected MCs, seems to be not the only mechanism by which these cells are able to interfere with the bacterial growth of enterococci.

Another extracellular mechanism employed by MCs in response to *E. faecalis* is degranulation. Recent studies have shown that degranulation leads to an effective killing of various Gram-positive as well as Gram-negative bacteria [183]. The blocking degranulation of MCs by cromolyn could restore bacterial growth, which was observed in the presence of MCs indicating an important role in the antimicrobial response of MCs during infections with *E. faecalis*. MC degranulation may be accompanied by the release of antimicrobial peptides such as cathepsin G or cathelicidins [88, 184], which are known to inhibit bacterial growth of various Gram-positive bacteria such as *S. pyogenes* or *S. aureus* [88, 185, 186]. An important antimicrobial peptide released by MCs is the cathelicidin LL-37 [88, 98].

The results presented in this thesis show that *E. faecalis* is very sensitive towards the anti-microbial effect of cathelicidin LL-37, indicating a potential major role of this anti-microbial peptide in the antimicrobial activity of MCs against *E. faecalis*. Electron microscopy examination revealed that *E. faecalis* exposed to either MCs or to LL-37 developed vesicle-like structures on the bacterial surface. Such vesicles have been described mainly for Gram-negative microorganism like *E. coli* [183], *Salmonella enterica* [187] or *Pseudomonas aeruginosa* [188] and some Gram-positive microorganisms like *S. aureus* [189]. The function of these vesicles is not yet clear but they may be related to bacterial responses towards environmental stress, which in this study might be produced by the anti-microbial compounds of MCs acting on the bacterial cell wall.

LL-37-treated *E. faecalis* showed morphological changes of the membrane. These vesicle-like formations are similar to structures found on *S. pyogenes* when they were treated with LL-37 [99]. The treatment of *E. faecalis* leads to a significant lower bacterial growth compared to an untreated control [158]. When *E. faecalis* is co-cultivated with MCs, similar vesicle-like structures can be observed [158]. This finding would be the first report of vesicle-like structures induced on the surface of enterococci by LL-37 [158] and might highlight the mode of action by which this antimicrobial peptide interferes with the growth of enterococci.

Taken together degranulation as well as the release of antimicrobials are another important extracellular control mechanism of MCs to limit bacterial growth of this pathogen. The bacterial factors involved in the induction of MCs degranulation after exposure to *E. faecalis*, however, remain to be determined in future studies. Common bacterial factors form Gram-

positive bacteria inducing degranulation of MCs are mostly exotoxins like the δ-toxin of *S. aureus* or the hemolytic pigment/lipid toxin form group B streptococci [190, 191]. Next to these bacterial factors there are several other inducers of degranulation of MCs including chemicals, drugs and environment factors [159, 192-194].

There are multiple direct and indirect pathways by which MCs can be selectively activated by pathogens including TLRs, co-receptors and complement receptors. TLRs are of central importance. Signaling via these receptors guides the immune system to produce an effective immune response to invading pathogens. It still is not well understood by which mechanisms TLRs are able to distinguish between a pathogen and a harmless commensal organism [195].
The recognition of bacterial pathogens by host cells is to a great extent mediated by the TLR/MyD88 pathway [196-198] while the classical activation of MCs is induced by interaction of dimerized IgE with the FcεRI receptor [199]. However, the interaction of TLRs with PAMPs also initiates the activation and degranulation of MCs [200].
The specificity of TLR recognition has been identified for several important PAMPs, including recognition of peptidoglycan (PGN), bacterial lipoproteins and zymosan by TLR2, double-stranded RNA by TLR3, LPS and heat-shock proteins (HSPs) by TLR4, flagellin by TLR5 and CpG motifs of bacterial DNA by TLR9 [201]. TLRs are expressed on many cell types including MCs. A critical role for TLRs in recognition of microorganisms or microbial products resulting in a complete and effective host defense has been established. *E. faecalis* expresses a variety of PAMPs that are recognized by TLRs [202, 203]. The precise TLR signaling and downstream events during the initial phase of infection with *E. faecalis* remains largely unknown, therefore, in this study one important focus was to shed light into which receptors are involved in *E. faecalis* interaction with MCs. MCs are more and more recognized to play an important role in the immune defense against bacterial pathogens, besides their traditional role in allergy immunity to parasites [204, 205]. The contributions of TLR-mediated mechanisms and subsequent effector functions of MCs during enterococcal infection are largely unknown. Beside the unclear state of TLR-mediated mechanisms involved in the response to enterococci, there is also only little knowledge about the composition of TLRs expressed by MCs. Twelve different TLRs are present in the genome of mice and ten in the human genome [69]. Using *ex vivo* MCs or MCs differentiated from bone marrow as well as cell lines, the composition of TLRs on MCs was described as a combination of TLR1-4 and TLR6-9 that are all present on BMMCs, while TLR5 and 10 was not detectable on these cells in several different studies [200, 206-211]. The involvement of

some TLRs in the immune response to enterococci have been demonstrated in recent studies, involving the recognition of enterococcal nucleic acids by the endosomal TLR7 and 9 in macrophages [212]. In the frame of this work immune responses mediate by TLR2, one of the most important TLR in terms of Gram-positive bacteria was investigated. This interaction is of particular interest, because it has been recently shown that TLR2 signaling in MCs is crucial for the effective control of other pathogens like *Francisella tularensis* [196].

Even though no difference in the association of *E. faecalis* to wild type or TLR2 $^{-/-}$ MCs could be detected, wild type MCs release significantly higher amounts of IL-6 compared to TLR2 $^{-/-}$ cells 6 h post-infection. Interestingly, no difference was found in the release of TNF-α, which is pre-stored in secretory granules and released upon different triggers. This finding is particularly unexpected, because TLR2 $^{-/-}$ cells display a significantly reduced degranulation response as measured by the release of β-hexosaminidase by knock-out cells compared to wild type MCs. Thus, suggesting a clear role of TLR2 signaling in the degranulation process. These findings can be explained by the fact that the release of distinct secretory granules subsets is highly regulated by different SNARE isoforms [213], directing the fusion of different cellular compartments in response to cellular signaling processes. Further studies have to demonstrate whether the impact of TLR2 really alters effects on the extracellular killing abilities of MCs in the course of *E. faecalis* infections *in vivo*.

Interestingly, it could be also demonstrated in the frame of this study that the formation of vesicle-like structures on the surface of *E. faecalis* is diminished if these cells are co-cultivated with MCs deficient for TLR2 or MyD88. This observation also highlights the role of TLR/MyD88 signaling for degranulation in general and LL-37 release in particular of MCs [158].

The formation of MCETs on the other hand was shown to be a TLR/MyD88 independent mechanism as demonstrated by the fact that MCETs formation of MCs in response to *E. faecalis* did not differ between TLR2 or even MyD88 knock out cells to cells of wild type [158].

In summary, this study provides the first experimental evidence that MCs exert antimicrobial activities against enterococci by extracellular mechanisms and thereby supporting a protective effect of MCs against this emerging pathogen. Furthermore, some details could be provided regarding the importance of TLR/MyD88 signaling in the immune response of MCs to *E. faecalis* and in particular in the degranulation of MCs triggered by *E. faecalis*.

Host defense against infection always requires an integrated response of both, the innate and the adaptive branches of the immune system. The innate immune defense system consists of many different cellular and humoral components that act in a coordinated fashion to prevent the manifestation of infection diseases. DCs are of central importance for initiating immune responses against invading pathogens. Next to MCs, dendritic cells (DCs) are also found on sites within the host that are exposed to the environment which makes them perfect sentinels for the recognition of pathogenic bacteria [105]. Therefore, another objective of this thesis was to elucidate the interaction of DCs with *E. faecalis*.

First, *in vivo* observations in this study using a GFP-expressing *E. faecalis* strain in a peritoneal infection model clearly demonstrated a close association of *E. faecalis* with DCs *in vivo* and second, a significant infiltration of these immune cells into the peritoneal cavity at early time points of infection.

There is only very limited knowledge available about the interaction of *E. faecalis* with DCs. It has been reported recently that *E. faecalis* cell surface-associated lipoproteins and the enterococcal polysaccharide antigen, which have been shown to be relevant for *E. faecalis* virulence in invertebrate infection models [214], also exhibit colitogenic tendencies in a susceptible mouse model, by activating DCs via TLR2 [214]. The loss of the *E. faecalis* lipoprotein signal results in reduced levels of secreted IL-12p40 by DCs. However, these DCs have been shown to be still able to reactivate colitogenic T-cells. Interestingly, *E. faecalis* lipoprotein-dependent induction of TNF-α and IL-6 secretion was completely abrogated in BMDCs from TLR2 $^{-/-}$ mice [214]. Other studies highlight the properties of probiotic *E. faecalis* strains to modulate the immune response of DCs towards a T_H1 response in a TLR dependent fashion, enhancing the immune response and shift the cytokine profile to the production of inflammatory mediators. The authors argue that probiotic *E. faecalis* strains could be used as a novel adjuvant strategy for oral or systemic vaccination [215].

All these findings underline the role of DCs as immune modulatory cells upon infection with *E. faecalis* and strengthen the importance to illuminate the cellular processes taking place during the encounter of DCs with enterococci. Therefore, a deeper knowledge about their implication for beneficial and/or detrimental aspects of immune processes elicited by these opportunistic bacteria is necessary.

The wide distribution of DCs throughout the body enhances their capacity to encounter invading microbes. During microbial invasion and tissue destruction, immature resting DCs, which have a unique recognition system to monitor danger signals are activated, and undergo a defined maturation process [104]. On the one hand activated and matured DCs lose their

antigen-capturing ability, but on the other hand they acquire antigen-presenting properties. These DCs migrate to the lymph nodes, where they are able to stimulate T cells [216]. Mature DCs also express high levels of MHC class II and costimulatory molecules such as CD40, CD80, and CD86. Furthermore they produce various cytokines such as IL-12 and TNF-α [217, 218] of which IL-12 is of central importance in the coordination of an efficient immune response [107].
In this study it could be also demonstrated that infection of wild-type DCs with *E. faecalis* resulted in a significant upregulation of cell-surface associated DC-activation markers as shown by the increased expression of costimulatory molecules such as CD40, CD80 and CD86 molecules.

To initiate the cellular responses, *E. faecalis* has to be recognized by DCs. Recognition of microbes by innate immune cells is mediated by PRRs via TLR receptors which main aspects have been already discussed in the section above.
To unravel the contribution of TLRs in the immune recognition and response of DCs to infections with *E. faecalis*, the requirement for the TLR associated adapter molecule MyD88 in maturation and production of cytokines by DCs after exposure to *E. faecalis* was investigated. MyD88 is the central adaptor molecule for responses several microorganisms or their components which are recognized by TLR2, TLR4, TLR5, TLR7, or TLR9. Some TLRs such as TLR3 and TLR4 signal via MyD88-independent pathways [219, 220]. The MyD88-dependent pathway was shown to be generally central for the host defense against microbial infections *in vivo* from a hole variety of different organisms such as *S. aureus* [221], *Listeria monocytogenes* [222] or *Toxoplasmam gondii* [223]. The resistance to *M. tuberculosis* on the other hand is affected largely through MyD88-independent pathways [224, 225]; these findings however document that the role of MyD88 during infection is very diverse and pathogen dependent. Not only devastating but also protective roles have been demonstrated for this central adapter molecule. It has been reported that MyD88-deficient mice are significantly more protected against the lethal effects of polymicrobial septic peritonitis [226] and also show less lethality if they are infected by a high inoculum dose of Group B streptococci than wild type mice with an intact MyD88 signaling pathway [227].
The beneficial effect of MyD88 displacement and also displacement of the TRIF pathway in experimental settings used in these studies seems to be based on the attenuation of a hyper inflammatory response and therefore accompanying with a reduced tissue destruction [228].

This might be also a very important issue in the context of enterococcal infections. These infections are often developing into a systemic septicemia with severe life-threatening conditions that is often initiated by a systemic inflammatory response to the microbe leading to impaired perfusion and possible multiple organ failure.
However, this study was also able to confirm that MyD88 signaling is essential for the activation process of DCs and furthermore for the production of IL-12 by DCs in response to *E. faecalis*.
IL-12 is a heterodimeric proinflammatory cytokine that induces the production of interferon-gamma and favors the differentiation of T_H1 cells and forms a link between innate resistance and adaptive immunity [229]. DCs and phagocytes have been shown to produce IL-12 in response to pathogens during infection [230]. Although several cell types including DCs and macrophages produce IL-12 after exposure to *E. faecalis* [231, 232], it is not clear which cell type is essential to initiate a protective immune response against infection with enterococci.

Although the precise mechanism by which IL-12 contributes to the innate immune defense against *E. faecalis* is not fully understood, studies in murine infection models have shown that the production of IFN-γ at the site of infection is in particular induced by IL-12. Moreover, the release of this cytokine was critical for mounting an effective innate immune protection [233]. Additionally, other studies have also highlighted the importance of IL-12 induced production of IFN-γ as essential for mounting an efficient defense response against polymicrobial infection via increased antimicrobial activity, nitric oxide production as well as increased phagocytic properties of innate immune cells like PMNs [234]. As demonstrated here DCs are recruited to the peritoneal cavity of mice infected with *E. faecalis* within less than one hour after infection, thus indicating that these cells are important contributors in the host defense towards enterococci most probably by the stimulation of phagocytic and microbicidal activities of innate immune cells through the production of IL-12 and accompanying local induction of IFN-γ. Murine infection models have also suggested that the production of sufficient levels of IL-12p70 early in infection provides a higher level of resistance to bacterial infections. This was demonstrated for example for the human pathogens *S. pyogenes* [113, 233, 235].
In this study it could be clearly demonstrate that the release of IL-12p70 as indicator for a full maturation status of DCs in the course of *E. faecalis* infection was strongly depended on a MyD88 based signaling processes, whereas the presence of TLR2 was found to be not essential, given by the fact that the production of IL-12p70 was not completely abolished in

DCs deficient in TLR2 expression. However, a significant delay in the release of IL-12p70 could be observed underlying the importance also of TLR2 signaling in initiating an efficient release of this important immunomodulatory cytokine. In agreement with the findings in these study previous investigations also suggested that the production of IL-12p70 by DCs is completely dependent on MyD88 and that loss of this adapter molecule totally abolished the production of this cytokine [114, 236].

After stimulation with *E. faecalis* matured DCs are not only releasing classical inflammatory cytokines, but also cytokines that are involved in the recruitment of phagocytes. These chemotactic active molecules are the CXC motif cytokines KC, responsible for neutrophil recruitment to the infection site and MIP2 responsible for the recruitment of macrophages [163, 237, 238]. In contrast to the observation made here other Gram-positive bacteria like *S. aureus* have been shown not to induce the release of KC and/or MIP2 in DCs [239], which also might argue for an important protective role of DCs in the course of enterococcus infections by contributing to high tissue levels of chemotactic mediators like KC or MIP-2. In future studies, it needs to be investigated if the ablation of the TLR/MyD88 signaling is protective in the course of enterococcal infections *in vivo*.

Another important feature of DCs is their phagocytic activity, which is decreasing with the process of maturation. These properties have been demonstrated already in the early 90s in studies using latex beads and zymosan particles which were internalized by DCs [240-242], but also apoptotic bodies [243], as well as microbes such as *M. tuberculosis* Bacille Calmette-Guérin (BCG) [244, 245], *S. aureus* [240], *Corynebacterium parvis* [241], Leishmania spp. [246] and *Borellia burgdorferi* [247]. It also has been shown that this intracellular niche is utilized by some pathogenic bacteria, such as *S. aureus* [109]. In that work it has been demonstrated that DCs do not contribute to a direct killing of *S. aureus*. Using live cell imaging the authors demonstrated a rapid increase in the number of intracellular bacteria over time as well as the escape of *S. aureus* into the cytoplasm of DCs followed by intracellular replication of the bacteria. For other Gram-positive bacteria like *S. pyogenes* a clear contribution to bacterial killing by DCs could be observed [113].

Using electron and fluorescence microscopy methods it could be demonstrated that murine DCs potently phagocytize *E. faecalis*. 6h post-infection, no bacteria were detected intracellularly arguing for a potent killing capacity of DCs against *E. faecalis*.

The lysogenic active compartment of many cells is the phagosome [161]. This compartment contains enzymes and an acidic luminal pH of 5.5-6. [248], but also a lot of lysosomal-

associated membrane proteins (LAMPs) [249]. To gain further insights into the intracellular localization of ingested bacteria, a LAMP-1 staining was performed using a GFP-expressing *E. faecalis* strain. Most enterococci within DCs were enclosed within intracellular compartments and co-localized with LAMP-1. It has been demonstrated earlier that the activation of the TLR signaling pathway by microorganisms is able to elicit phagocytosis at various steps of the internalization process and moreover phagosome maturation [160]. Interestingly, in contrast to the important impact of the TLR/MyD88 signaling pathway on maturation and cytokine release neither phagocytosis nor intracellular killing of *E. faecalis* by DCs was affected by this pathway. BMDCs originated from mice deficient in TLR2 and MyD88 showed no difference in the number of internalized bacteria and also no loss of intracellular killing ability compared to wild type cells.

It has to be mentioned that in general the impact of TLR deletion on *in vivo* phenotypes of microbial infections is often not severe. A good example in this aspect is that LPS is a potent inducer of TLR4 signaling. However, mice deficient in TLR4 expression do not display a higher degree of susceptibility to infection with Gram-negative pathogens [250]. This finding was also observed for other bacteria like *L. monocytogenes*, which is not inducing higher susceptibility levels in TLR2 deficient animals [251]. These findings emphasize the complexity of immune recognition of invading microorganism by various immune cells. However, the data presented in this study underscores a potential protective role of DCs in the immune defense against *E. faecalis* and indicates a coordinated interplay between MyD88-independent events like phagocytosis and direct bacterial killing of enterococci as well as MyD88-dependent processes that involve DC maturation and cytokine release.

Besides resident immune cells representing the first barrier of the host innate immune response, like MCs and DCs, the endothelium represents an important first contact point for invading microorganism. In particular, biofilm-associated diseases like endocarditis established by enterococci as well as blood borne infections, like bacteremia leading to sepsis and multiple organ failure, involves the response of endothelial cells.

Cultured human umbilical vein endothelial cells (HUVEC) have been intensively used to study the interaction of Gram-positive and Gram-negative bacteria with the endothelial barrier. Nevertheless, there are only a few studies conducted aiming to establish the relationship of *E. faecalis* with endothelial cells. The emergence of high level aminoglycoside resistance (HLAR) and vancomycin-resistance gene cluster carrying (VRE) *E. faecalis* strains has limited the success of antibiotic treatment in case of infections caused by this organism

and highlighted the importance to study the interaction of these bacteria with their target tissues [6, 14].

Therefore, the aim of the last part of this thesis was to gain deeper insights of how *E. faecalis* is interacting with this important biological barrier, because one of the most concerning disease manifestations caused by enterococcal species is endocarditis. This disease involves the interaction of enterococci with endothelial cells of the middle heart blood vessel system or on heart valves leading to biofilm formation and subsequently eventually to endocarditis [10]. HUVEC are used as an *in vitro* model to study initial bacteria-endothelial interactions. The mechanisms by which enterococci cause endocarditis are still poorly understood and research in this field is mainly focusing on the bacterial factors involved in the formation of biofilms. It has been shown that *E. faecalis* is able to adhere to eukaryotic cells by the properties of its surface molecules that interact with host cell receptors [180, 252-254].

Many pathogens hide inside cells to protect themselves from recognition by cells of the host immune defense like neutrophils or macrophages. A few studies indicated also an intracellular stage for *E. faecalis* in macrophages [164] and epithelial cells [255]. *E. faecalis* were found within the intracellular milieu regardless of the fact that *E. faecalis* is considered to be an extracellular pathogen. It also was observed that these bacteria adhere to and invade HUVEC cells and hide inside large-scale vacuoles [256]. The ability of *E. faecalis* strains and clinical isolates to enter cultured HUVEC was investigated by these authors and they have shown that under the conditions they used *E. faecalis* was able to enter and survive within HUVECs. The ability to adhere and invade endothelial cells was shown and documented also for other Gram-positive bacteria like *S. aureus* and viridian group streptococci [257-260]. On the other side other Gram-positive bacteria have been shown not to invade the endothelial barrier, like *B. subtilis* or some *S. gallolyticus* strains [256, 261]. However, the MOI used in the study of Millan and colleagues was relatively high reaching the lowest level at an MOI of 100:1. Furthermore, the bacteria were sonicated to avoid bacterial clumping and chain formation. In this work only adherence, but no internalization of *E. faecalis* into endothelial cells was observed. This discrepancy might result from cellular variability of endothelial cells used and is more important from different experimental settings using more physiological conditions for infection at an MOI of 10:1 and without destroying the natural occurrence of bacterial chains. Most studies demonstrating *E. faecalis* inside cells have used cell types with high phagocytic properties like macrophages [262] or neutrophils [56]. Therefore, it can be argued that appearance of *E. faecalis* within host cells depends on active phagocytosis and seems to be not a result of active invading processes induced by the bacteria [164].

A typical response of many host cells to bacterial infection is the release of proinflammatory cytokines and mediators. One prominent inflammatory cytokine is IL-8, which is released also by endothelium cells [133, 136, 263]. This cytokine is of central importance for recruitment of neutrophils to the site of infection as well as for the degranulation of neutrophils, which is leading to exocytosis, *e.g.* histamine release. Endothelial cells are able to store IL-8 in their storage vesicles, the so called Weibel-Palade bodies [264]. In target cells, IL-8 induces lots of physiological processes being involved in cell migration and phagocytosis processes, like increase respiratory burst and Ca^{2+} influx. Different studies also have shown the importance of IL-8 and its role in infective endocarditis [265, 266].
In this study it was described for the first time, that *E. faecalis* is able to diminish the release of IL-8 by endothelial cells. This finding is of interest, because high levels of IL-8 release have been reported to be associated with infective endocarditis [267] and have been even linked to fatal outcome [265]. Interestingly, this finding is consistent with staphylococcal infective endocarditis, in which the level of IL-8 was also reduced [266]. Jijon *et al*. showed in their study that an inhibition of extracellular signal-regulated kinase (ERK) or p38 leads to a reduction of IL-8, but not to a reduction of ICAM-1 surface expression [268]. Other study showed that wild-type *L. monocytogenes* are inducing high levels of IL-8, whereas *Listeria* strains deficient in the release of listeriolysin O- or internalin B- as well as other pathogenic listeria species like *L. innocua*, substantially lack the induction of IL-8 production in endothelial cells [269]. The bacterial factors being responsible for the reduced release of IL-8 in the case of *E. faecalis*-triggered infection of the endothelium remains to be elucidated in future studies, potential candidates could be the cytolysins, cytolysin A and enterolysin A produced by *E. faecalis*.

The response of endothelial cells to bacterial infections is often associated with the up-regulation of receptors involved in process of leukocyte extravasation ICAM-1, E-selectin and VCAM-1 [263, 270], which are induced by numerous inflammatory cytokines, in particular IL-1β, TNF-α or IL-6 [168, 271, 272]
Up-regulation of ICAM-1 is induced by cell wall and membrane components of Gram-positive bacteria like staphylococcal PNGs in various cells, *e.g*. fibroblasts, which also release high levels of inflammatory cytokines like IL-6 and IL-8 [273]. In the work presented here a significant reduction in the expression of one of these important surface receptor on endothelial cells was observed. ICAM-1 could not only be detected on the level of

transcription but even more important not on the level of translation. This effect was not limited to the ICAM-1 receptor alone but occurred also for E-Selectin and VCAM-1. The functionality of the cells under the experimental conditions used was not affected as could be demonstrated by the fact that IL-1β, a strong inducer of the expression of leukocyte extravasation receptors [274, 275], showed a normal and strong upregulation of this receptor family.

This finding is of importance, because it suggests together with the diminished levels of IL-8 induced by this pathogen that *E. faecalis* is capable to manipulate the response of the endothelium for its own benefit, *e.g.* by impairing the inflammatory immune response elicit by endothelial cells. A consequence of this manipulation might be for example that the formation of biofilms induced by enterococci and the colonization of heart valve and blood vessel tissue is alleviated. This process can evoke also consequences in the manifestation of infective endocarditis induced by *E. faecalis*. The importance of the findings within this study is further strengthened by the fact that the cushioned inflammatory response of HUVECs cells during infection with *E. faecalis* is not donor- or cell source-depended, as demonstrated by the use of endothelial cells from different origins.

The molecular mechanisms by which microorganisms or their products are able to manipulate the expression of surface receptors involved in leukocyte extravasation are still poorly investigated.

The reduced expression of VCAM-1 in LPS-induced inflammation for example is known to be regulated through the novel anti-inflammatory cytokine IL-35, which inhibits the expression of this receptor via the AP-1 signaling pathway [276]. Another important factor in the regulation of VCAM-1 is the transglutaminase2 (TG-2) that has been documented to be necessary for the expression in an NFκB-depended manner. By silencing TG2 using inhibitory RNAs (iRNAs), the expression of VCAM-1 was inhibited [277]. It remains to be investigated, if these mechanisms are employed by enterococcal strains.

Up-regulation of E-Selectin is induced in general through NOD1, NOD2 and TLR signaling pathways during bacterial infections [278] and is often used as a biomarker for infective endocarditis [279]. Under the experimental settings used in this study no significant upregulation of E-Selectin could be observed, which might exclude E-Selectin as biomarker for infective endocarditis induced by *E. faecalis*.

It has been observed that endothelial cells develop a tolerance in terms of E-Selectin expression after continuous stimulation with LPS. Therefore, it might be possible that cell

wall components of enterococci have the property to induce an anergy in the expression of this receptor family, an aspect that would be of interest for further investigations.

In summary, this wok presents evidence that *E. faecalis* is able to manipulate the inflammatory response of the endothelium by diminishing the release of IL-8 and interfering with the surface expression of important receptors involved in leukocyte extravasations in particular ICAM-1 and VCAM-1. This offers a new and so far unknown pathogenic property of enterococci which might allow the bacteria to circumvent the host immune response and enables the bacteria to colonize the endothelium more efficiently.

5. Summary

Enterococcus faecalis has emerged as an important cause of life-threatening bacterial infections in the hospital environment especially in ICUs. Enterococcal infections are the third most common cause of hospital acquired bacterial infections in the US and in Europe. Enterococci have an intrinsic resistance driven by the accumulation of mobile genetic elements to many clinically used antimicrobials agents, making them difficult to treat and therefore important nosocomial pathogens.

Surprisingly very little is known about how this opportunistic pathogen interacts with the host immune system and modulates its responses. In addition, molecular and cellular processes involved in his host-pathogen interaction remain largely unknown *e.g*. how bacteria enter sterile sites of the body to cause disease. Only a few studies have investigated the interaction of enterococci with professional phagocytes like macrophages and PMNs. Therefore, one major focus of the thesis was to explore how other important and so far neglected sentinel cells of the innate immune system, like mast cells (MCs), dendritic cells (DCs) and also the endothelial barrier, representing important first counterparts of bacterial pathogens with important immunomodulatory functions respond to infections with *E. faecalis*. Classically MCs are known for their role in allergic reactions and parasite affection, but only a few efforts were undertaken to unravel their role during bacterial infection with in parts contradictory results, showing beneficial as well as detrimental functions of MCs in the course and outcome of infectious diseases. This thesis clearly demonstrates a protective role of MCs against *E. faecalis* mediated by extracellular processes: (1) the release of so called mast cells extracellular traps (MCETs) that are able to ensnare and kill bacteria and (2) by the release of pre-stored antimicrobial peptides that are directly antimicrobial.

The cathelicidin LL-37 was identified as the antimicrobial agent responsible to induce cell wall stress and vesicle formation on the bacterial membrane of *E. faecalis*.

Furthermore, the importance of the TLR/MyD88 signaling pathway in the response of MC to *E. faecalis* could be strengthened by using MCs deficient in the expression of MyD88 and TLR2.

DCs in comparison to MCs were found to be able to phagocytize and kill *E. faecalis.* Furthermore, they release a cytokine milieus consisting amount of important immunomodulatory molecules like IL-12, but also mediators like KC or MIP2, belonging to the CXC chemokine family. KC and MIP2 are of central importance for the recruitment of PMNs and macrophages, both innate immune cell types essential for clearance of *E. faecalis*.

Interestingly, it could be determined that the TLR2/MyD88 pathway is also important for the induction of cell maturation and the release of cytokines and chemokines underlining a key role of this PRR sensing pathway in the immune response towards enterococci.

Upon entry into the blood stream, *E. faecalis* faces the endothelial cell barrier of the host, separating the blood from deeper tissue layers. Another important function of endothelial cells is the production of cytokines and chemokine during bacterial infections. Additionally, the endothelium is involved in a process called leukocyte extravasation which is taking place at the endothelial layer and is mediated by the expression of endothelial surface receptors like ICAM-1, E-selectin and VCAM-1. This study provides for the first time evidence, that *E. faecalis* interferes with the expression of these receptors and therefore blocks adhesion and transmigration of leukocytes through the endothelial barrier.

These findings in combination with others findings like the diminished IL-8 response strengthen a manipulation of the endothelium by *E. faecalis.* This might be a new and interesting process by which these bacteria evade immune responses by the host and facilitate colonization and infection of the endothelial barrier. Factors produced by enterococci to achieve this manipulation warrant further investigations.

In summary, the study presented here provided a novel insight into the innate immune mechanism involved in the response to this emerging pathogen. It highlights the molecular and cellular processes involved in the natural resistance to these microorganism as well as the defense strategies utilized by *E. faecalis* in the conversion of an opportunistic commensal into a pathogenic organism that is able to colonize the host and induce potentially life threatening diseases.

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Appendix

Abbreviations

°C	degree Celsius
3´	three prime end
5´	five prime end
APC	Allophycocyanin
APCs	antigen-presenting cells
BEA	bile esculin agar
BSA	bovine serum albumin
BHI	brain heart infusion
BMMC	bone marrow mast cells
BSS	balanced salt solution
C3	complement component 3
CCL	chemokine (C-C-motif) ligand
CD	cluster of differentiation
cDNA	copy deoxyribonucleic acid
CFU	colony forming units
CO_2	carbon dioxide
Comp 48/80	Compound 48/80
CpG	cytosine-phosphate-guanine
Cromolyn	5.5′-(2-hydroxypropane-1.3-diyl)di(oxy)di(4-oxo-4*H*-chromene-2-carboxylic acid)
CTR	control
CXCL	chemokine (C-X-C-motif) ligand
DAMPs	danger-associated molecular patterns
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DTNB	5-(3-Carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid
EBM-1	Endothelia Basal Medium-1
EBM-2	Endothelia Basal Medium-2
ECM	Endothelia cell medium
EDTA	ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*

EGM-1	Endothelia Growth Medium-1
EGM-2	Endothelia Growth Medium-2
ELISA	enzyme linked immunosorbent assay
et al	*et alii* (Latin ‘and others’)
FACS	Fluorescence-activated cell sorting
FCS	fetal calf serum
FIG	figure
FITC	Fluorescein isothiocyanat
g	gram
GFP	green-fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour
h.i.	heat-inactivated
H_2O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FCS	fetal calf serum
IC	intracellular
IFN	interferon
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
l	liter
LPS	lipopolysaccaride
LTA	lipoteichoic acid
log	logarithm
M	molar
MCET	mast cell extracellular trap
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
MQ	MilliQ

MyD88 $^{-/-}$	diploid *myd88*-gene knock-out
*m*SCF	*murine* stem-cell factors
MSCRAMM	Microbial surface component recognizing adhesive matrix molecule
NaCl	sodium chloride
NaN_3	sodium azide
NEAA	non-essential amino acids
NET	neutrophil extracellular traps
ng	nanogram
nm	nanometer
NOD	non-obese diabetic
NF-κB	nuclear factor-κB
OD_{600nm}	optical density at 600 nm
ON	over night
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PBST	phosphate buffered saline plus Tween20
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
pg	pictogram
PRR	pattern-recognition receptor
P value	probability value
RIG-1	retinoic acid-inducible gene 1
RNA	ribonucleic acid
ROS	reactive oxide species
rpm	rotations per minute
RPMI	Roswell park memorial institute medium
RT	room temperature
SD	standard deviation
TAE	Tris-acetate-EDTA
TF	tissue factor
TGF	transforming growth factor
TLR2 $^{-/-}$	diploid *tlr*2-gene knock-out
TMB	3.3'.5.5'-tetramethylbiphenyl-4.4'-diamine

TPA	12-O-tetradecanoylphorbol-13-acetate
Tween-20	Polyoxyethylene Sorbitan Monolaurate
WT	wild type
α	alpha
β	beta
γ	gamma
ε	epsilon
κ	kappa
x g	gravitational acceleration
µg	microgram
µl	microliter
µm	micrometer

Illustration directory

Table directory

Table 9. Gene profile of virulence factors in *Enterococcus faecalis*

internal name	disease pattern	source	*ace*	*as*	*clyA*	β-hemolysis	*elyA*	*esp*	*ebpA*	*ebpB*	*ebpC*	*efaA*	*gelE*	*hyl*
***E. faecalis* 1**	blood	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	+
***E. faecalis* 11**	blood	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	-
***E. faecalis* 13**	blood	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 15**	blood	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 18**	blood	Hospital BS	+	+	-	-	+	+	+	+	+	+	+	+
***E. faecalis* 20**	blood	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 5**	blood	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	+
***E. faecalis* 8**	blood	Minneapolis	+	+	+	+ +	-	+	+	+	+	+	+	+
***E. faecalis* 2**	bone	Hospital BS	+	+	+	-	-	-	+	+	+	+	+	-
***E. faecalis* 14**	endocarditis	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 19**	endocarditis	Hospital BS	+	+	+	-	+	-	+	+	+	+	+	-
***E. faecalis* 21**	endocarditis	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	+
***E. faecalis* 29**	endocarditis	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	+
***E. faecalis* 3**	endocarditis & sepsis	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 10**	AHJ transplantation infection	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	+
***E. faecalis* 12**	AHJ transplantation infection	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 22**	AHJ transplantation infection	Hospital BS	+	+	+	+	-	+	+	+	+	+	+	+
***E. faecalis* 4**	AHJ transplantation infection	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 6**	AHJ transplantation infection	Hospital BS	+	+	+	-	-	+	+	+	+	+	+	-
***E. faecalis* 7**	AHJ transplantation infection	Hospital BS	+	+	-	-	-	+	+	+	+	+	+	-
***E. faecalis* OG1RF**	lab strain	DSMZ	+	-	-	-	-	-	+	+	+	+	+	-

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